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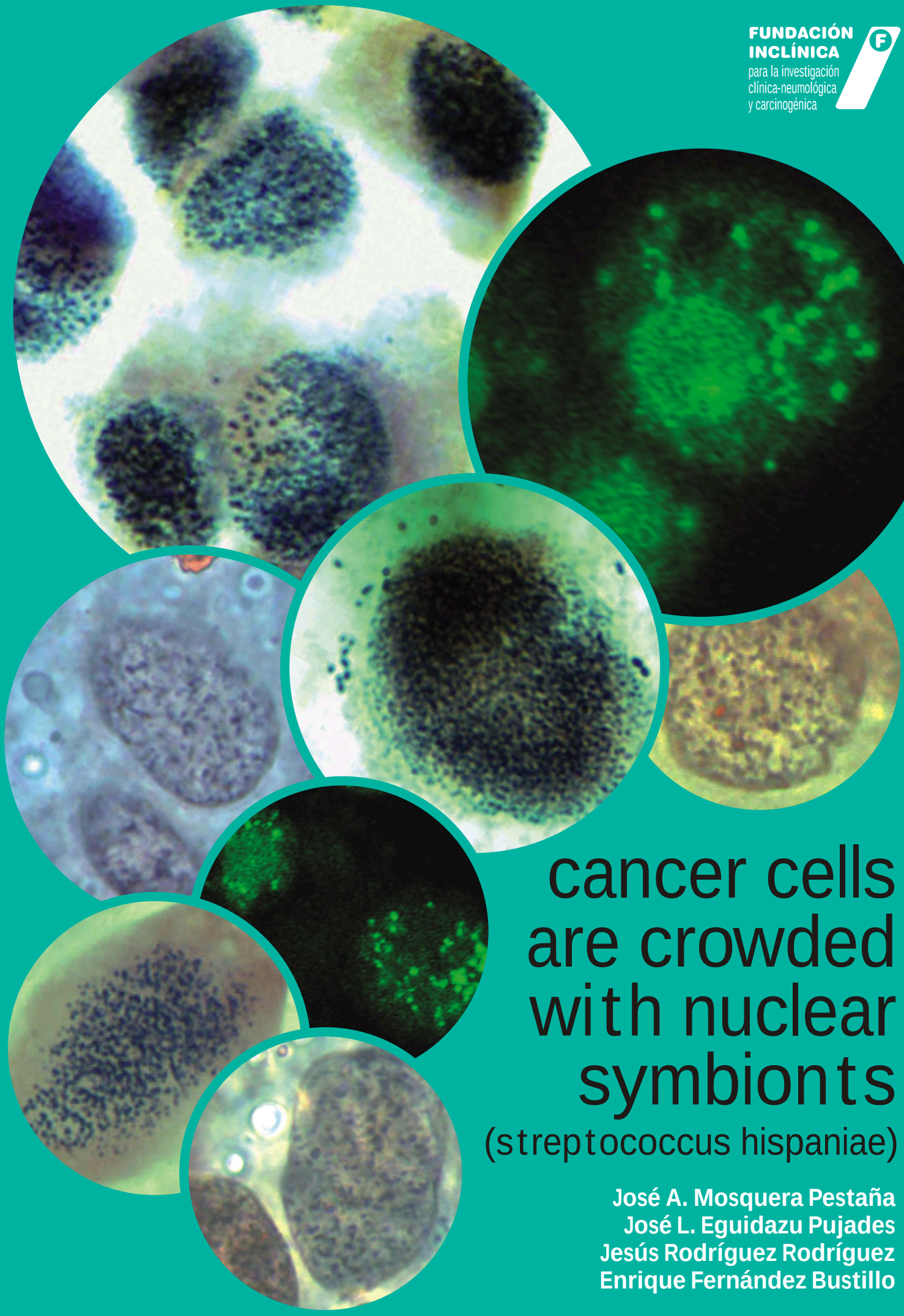

cancer cells are crowded with nuclear symbionts

(streptococcus hispaniae)

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To
Our:

Sons and daughters,
Parents,
Admirable wives,
Inclínica members,
National Institute of Silicosis' workers

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PREFACE

Since 1985, a group of experts in the field of health, consisting of physicians specialized in pneumology, pathology and thoracic surgery, as well as engineers and other technicians of the field, have developed in the Principado de Asturias, moved by an altruistic spirit and the love for their profession, two important research lines of a clinical nature which are very close to their daily performance: the first, on the presence of protozoa in the sputum of the majority of asthmatic patients; the second, in connection with tumor cells in the lung.

In recognition of the ambition and the effort undertaken by this group of researchers, a number of entrepreneurs and professionals have contributed to the creation, in 2000, of the Fundación Inclínica para la Investigación Clínica-Neumológica y Carcinogénica (Inclínica Foundation for Clinical-Pneumological and Carcinogenic Research), a Spanish, non-profit making, small-sized entity aimed at ensuring the continuity of research projects through its financial, technical and organizational support.

Fundación Inclínica started from an initial consideration which determines its philosophy, structure and functioning. The starting point is related to a fragmented knowledge, imposed by a tight protocolization of research activities which sometimes generates important dysfunctions in addressing practical clinical problems. The promoters of Fundación Inclínica did not question the principles of specialization, quite the contrary, having acknowledged their value, they realized there was a wide field of action to cover in which small entities could make a significant contribution. To serve this space, on the one hand, the creation of multidisciplinary teams is required wherein the clinician, who possesses valuable information from the daily observation of cases, shall be the center of a group which also includes scientists specialized in both basic and applied sciences working with him or her in the laboratory within the hospital itself. Thereby, the medical observations would be supported by those of experts in biology and other fields of knowledge. This would provide a point of view of the problems which physicians usually lack. On the other hand, the management of the entity funding that team must be entrusted to professionals from the business world.

As a result, a multidisciplinary human team has been established, composed of researchers and managers who, altruistically, devote part of their daily efforts to the achievement of certain general interest goals in connection with clinical research. Taking good account of the Hipoccratic maxim, ars longa, vita brevis, the main virtue of this team is that of patience, which is not groundless, but founded on two cornerstones: the first one, the belief that science knows no well-trodden paths nor is required by imperative deadlines; the second one, that of confidence in the scientific quality of the researchers involved and the seriousness of their work, which is abundantly proven by the consistency of their professional résumés and the permanent contrast of their work with the breakthroughs of the scientific community.

Furthermore, Fundación Inclínica knows that the day to day of scientific knowledge is characterized by failure, as the space covered over months or years often only leads to closing the door to a hypothesis, a method, a protocol which had to be excluded. In biopathology in particular, any really original work requires many years of seeding and, typically, scarce harvesting, if measured in terms of successful results. The book that comes out today is a good example thereof. The team at Fundación Inclínica started to research on the origin of tumors more than twenty years ago and their results have provided no more than two communications to medical forums. During the last four years, however, and after countless failed tests, the path that has led to this publication has been discovered, which reports very consistent findings not previously observed and which we believe will open new and interesting fields to cancer research.

At Fundación Inclínica we feel proud of the work done but, above all, we are deeply grateful to the numerous companies, institutions and individuals that have believed in the project and have collaborated directly in it, always altruistically. First of all, we would like to thank the Government of the Principado de Asturias, through its Consejería de Salud, with which Fundación Inclínica maintains a cooperation agreement that entitles this entity to use spaces thereof, for its own laboratories, as well as the many employees at the Instituto Nacional de Silicosis in Oviedo and the Hospital Universitario Central de Asturias, for their constant encouragement and support.

Secondly, the companies and individuals that regularly provide financial collaboration without any compensation: Asturcontinental de Edificaciones, S.L. (Mr. Jorge Fernández Vallina and Mr. Fernando Nuño Pérez); Mr. Evaristo Luis Bango Escacho; Caja de Ahorros y Monte de Piedad de Madrid; Construcciones Candanedo, S.A. (Mr. Emilio León Candanedo); Construcciones José Luis, S.A.; Construcciones y Promociones Coprosa, S.A. (Mr. Sabino Iglesias Vega); El Caleyo Derivados del Cemento, S.A.; El Caleyo Nuevas Tecnologías, S.A.; Explotaciones Mineras Solís, S.L.; Fontela Gestión y Edificación, S.A.; Ms. María del Pilar García Giraldo; Mr. Juan García González; Gestionalia Externalización y Servicios, S.A.; Gómez Oviedo, S.L. (Mr. Antonio Luis Gómez Aller); Hormigones El Caleyo, S.A.; Hormigones La Caridad, S.A.; Hoteles y Turismo del Principado, S.A.U. (Mr. Manuel Cosmen Adelaida); Industrias y Servicios de la Construcción, S.L. (Mr. Alberto Martínez Arroyo); Jesús Martínez Álvarez Construcciones, S.A. (Mr. Francisco Martínez Gómez); Proyectos, Construcciones e Interiorismo, S.A. - Procoin, S.A. (Mr. Constantino Martínez Pérez); Promociones Coto de los Ferranes, S.L.; Residencial Peñas de Europa, S.A. (Mr. Luis Priede Díaz); Sacejo Promociones y Construcciones, S.A.; S.A. Tudela Veguín (Mr. José Antonio Muñiz); Avilés' volunteers of Alcoa Foundation; and employees of the Fundación Laboral de la Construcción del Principado de Asturias. Thirdly, and of great importance to Fundación Inclínica are: the Fundación Laboral de la Construcción del Principado de Asturias, an entity that has constantly provided our foundation with human resources and support material; Mr. Ricardo San Marcos de la Torre, for his permanent technical assistance in the economic and taxation fields; Mr. Heliodoro Menéndez Alegre, who created the foundation's website; and Mr. Ignacio del Cueto Álvarez, responsible for the design of the website and all of the entity's publications.

Finally, I would like to personally thank all the trustees of Fundación Inclínica who have participated in the project from the very beginning or at successive stages. In addition to the authors of this work (Mr. José Antonio Mosquera Pestaña; Mr. José Luis Eguidazu Pujades; Mr. Jesús Rodríguez Rodríguez and Mr. Enrique Fernández Bustillo, who were assisted in the English version of the text by Ms. Olaya Astudillo Fernández; also, by Ms. Kirstie Bicknell and Mr. Daniel F. Celemín), Mr. Andrés Ribas Barceló; Mr. Juan Galán Fernández; Mr. Manuel García Rubio; Mr. Rafael Martínez Girón; Mr. Tomás Moraleda Roncero; and, as non-trustee counselor, Mr. Armando Adeba García, have been fundamental people. I must also note the laudable work of the biologist Ms. Verónica García Díaz, particularly devoted to the culturing of tumor cells. Last but not least I would like to pay tribute to the human quality of those who were trustees of the entity, Mr. Gerardo Collantes Martínez and Mr. Adolfo Galán Salvador, unfortunately deceased, whose enthusiasm is a permanent stimulus to continue with our work.

With the edition of the present study, Fundación Inclínica closes a stage of intensive and silent work, but also a fruitful one. We are confident that this shall only be the first step in a hitherto unexplored path, to which new supports and projects shall join.

Serafín Abilio Martínez Fernández
President
Fundación Inclínica

INTRODUCTION

The title of this book summarizes the main message of this study: malignant neoplastic cells are crowded with bacterial endonuclear symbionts.

For the readers that wish to read quickly this monograph, it is recommended to read Chapter 1, in which the most relevant information of every chapter is summarized.

For further information about methods, results, and a more extensive discussion, the reader should consult the specific chapters.

This report was designed to follow the idea of a proverb by Baltasar Gracian: *"Little, if good, twice as good, and even bad, if short, not as bad".*

CHAPTER 1

SUMMARY: MALIGNANT NEOPLASTIC CELLS ARE FILLED WITH BACTERIAL ENDONUCLEAR SYMBIONTS (STREPTOCOCCUS HISPANIAE)

This summary shows the most important facts described in this book.

Cocci that stain with lacto-orcein dye can be found inside malignant cells (Chapter 3); they are present in most resting cells, but not in those undergoing mitosis (Chapter 4). After tumor mechanical compression, these cocci are partly released outside the cell, and they are visible with Gram staining (Chapter 5). The positive Gram staining indicates that they are neither artifacts, nor a result of chromosome pulverization, since this staining indicates the presence of a cell wall that cannot come from eukaryotic cells such as tumor cells. The Gram-stained smears in 31 successive samples of lung neoplasms and 33 non-tumoral controls confirm that tumor cells are filled with Gram (+) cocci, about 100 cocci per cell, which enlarge and distort nuclei and cytoplasms (Chapter 6). This massive storage of bacteria in neoformation cells, but not in non-malignant lung cells, is present in most frequent histological cell types (squamous-cell carcinomas and adenocarcinomas), but is also found in less common epithelial tumors, metastatic cancer, and connective and lymphatic neoformations (Chapter 7). The further staining with Syto and Propidium iodide demonstrates the presence of living round bacteria in the interior of neoplastic cells. These bacteria are alive, even when the neoplastic cells are dead, indicating that they contain nucleic acids that are protected by an unharmed cellular membrane, which is different from the one of the host cancer cell.

Chapter 8 describes the morphological variability of tumors (the different patterns of fluorescence) compared with the uniform aspect of benign cells. Nearly half of the tumor cells are small and have thick fluorescent density, whereas the other half contain numerous bacterial clusters and/or multiple nuclear divisions, which fill the cell, and the cell diameter doubles in size, in comparison with the other cells. The new nuclei have the same appearance as small dense cells. Almost 6% of cells obtained within few minutes after surgical procedure are dead, but filled with living cocci, and with areas of nuclear destruction. This suggests that these cocci are endonuclear symbionts, with strong vitality and aggressive behavior.

Chapter 9 shows how few bacteria could be cultured from 22 successive samples of resected tumors and 24 of normal lung tissue. Bacteria grew poorly, and without significant differences between cultures coming from tumors or normal lung tissue, despite the massive presence of living Gram (+) cocci inside neoformative cells (living or dead).

Genetic analysis by sequencing the 16S rRNA gene and MALDI-TOF mass spectrometry, failed to identify any bacteria, despite the great presence of diplococci, tetrads or chains of Gram-positive organisms in tumoral cells. *Streptococcus sp* fits the characteristics shown by the three types of staining performed in this study: lacto-orcein, Gram, and Syto-Propidium iodide (Chapter 10). All data described in this book indicating the close and permanent colonisation of *Streptococci* inside tumor cells fulfils the criteria specific to endonucleosymbionts. Chapter 11 revisits the extraordinary widespread and fantastically complex biology of endosymbiotic bacterial mutualism in host eukaryotic cells, mainly in insects and protozoa. These bacteria cause pleiotropic effects on nuclear size, morphology, and aberrant mitosis, together with changes in growth, development, metabolic homeostasis, fecundity, toxic resistance, chemical products, and lifespan of the host.

The demonstration of the presence of these intranuclear cocci in both living and dead cancer cells shows that these organisms are aggressive endonuclear symbionts. *Streptococcus sp* increase and deform nuclei, as *Hollospora obtusa* does to the nuclei of *Paramecium caudatum*, and as the symbionts of *Vorticella* and *Paramecium trichum* cause destruction of their chromatine. Moreover, anomalous mitosis, similar to that occurring in tumors, can be seen with endonuclear symbiosis, such as in the nuclei of *P caudatum* carrying Kappa particles. Moreover, tumors are an impressive source of chemical substances, as are other organisms containing symbionts, and they induce widespread, complex changes known as paraneoplastic syndromes (Chapter 12). And finally, the causal role of *Streptococcus* in malignant tumors is discussed in Chapter 13.

We propose the name *Streptococcus hispaniae* to designate this new specie.

CHAPTER 2

BACKGROUND AND NEED FOR A PROSPECTIVE STUDY

1. Background

The aggressive behavior of tumor cells against normal cells promotes their growth and dissemination, to the point of predominating in many organs. Other models in nature show the destructive character of an organism against its neighbors that enhances its superiority.[1] Wellknown examples exist among protozoa, especially *Paramecium tetraurelia*, which, when carrying bacteria from genus *Caedibacter*, acquire a killer trait that destroys other strains of paramecia that do not contain the endosymbionts. This way, they propagate in their own ecological niches. These bacteria-containing protozoa share other characteristics with neoplastic cells: they have similar predisposing conditions, such as exposure to radiations and to chemical products; they also have nuclear similarities. When bacteria live inside the nuclei of protozoa (endonuclear symbionts), the nuclei become hypertrophied, lose their normal shape, and have anomalous mitosis. These characteristics are gold standard findings in tumors and are even used for their diagnosis.[2]

2. Aim

The aim of this systematic and prospective study was to look for the presence of endonuclear symbionts inside lung tumor cells, immediately after the surgical removal of the sample, and to compare these findings with benign cells adjacent to the neoformation obtained during the same surgical procedure.

3. Samples

From July 2008 to July 2012, 76 samples from suspected tumors and benign neighboring lung tissue, were collected immediately after surgical procedures at the Hospital Central de Asturias, Oviedo, Spain. Of the 76 samples, 75 were confirmed to be tumors and one (case 44) was an inflammatory pseudotumor (Table 2/1). The other neoplasms were: 34 (44.7%) squamous-cell carcinomas, 31 (40.8%) adenocarcinomas, four (5.3%) large-cell neuroendocrine carcinomas, two (2.6%) pleomorphic sarcomas, one (1.3%) primary pulmonary B lymphoma and three (3.9%) metastatic cancer, (Tables 2/1 and 2/2). According to TNM categories, the tumors were classified as follows: 13 (17.1%) were T1, 43 (56.5%) were T2, 14 (18.4%) were T3, and 6 (7.8%) were T4 neoformations (Tables 2/1 and 2/2). The mean largest diameters of the tumors resected was 4.7 cm (1SD 2.1) by 3.2 cm (1SD 1.2) with an area of 22.1 cm^2 (1SD 25.5). Of the 76 patients, 53 (69.7%) had a lobectomy, 16 (21.1%) had a neumonectomy, and 5 (6.6%) were treated with an atypical resection. All the 76 patients had a median age of 63.9 (1SD 10) years and 60 (78,9%) were men (Table 2/2).

4. Statistical methods

All data obtained were stored in an Excel® spreadsheet. We obtained from these data several indices and derived variables, and subsequently analysed these variables by means of the statistical packages SPSS® and STATCALC.

1 Hawking S, Mlodinow L. *El Gran Diseño.* 1st ed. Barcelona: Critica SL, 2010: 21.

2 Muller Km. *Lung cancer morphology.* In: Spiro SG, ed. Carcinoma of the lung. Sheffield: European Respiratory Society, 1995: 50–71.

We described the quantitative variables by their mean, standard deviation, maximum and minimum values, and the number of data used in their obtention. We described the qualitative variables by their frequency and their percentage of occurrence.

The analysis of quantitative variables versus dichotomic or multichotomic qualitative variables was carried out using the Student's t-test for the differences between means and the ANOVA model, after having tested variance homogeneity with the Levene's test. Whenever significant differences were found using ANOVA, multiple comparisons were also done with the Bonferroni's test. Where the variable analysed did not meet the parametrical test, the equivalent non-parametric tests were used: Mann Whitney and Kruskal-Wallis tests. In all cases, H0 (the null hypothesis) was rejected if $P < 0.05$.

For the analysis of the quantitative variables among themselves, the Pearson's correlation coefficient was calculated, showing for every pair of variables the value of r, the sample size n and the probability of error P when affirming that a significant correlation exists between those variables.

The relations between quantitative variables were examined through the analysis of 2×2 or 2×n contingence tables and by calculation of χ^2 values either directly or, where data was insufficient, carrying out the Yates' correction and, in the case of 2×2 tables, the Fisher's exact test. Whenever the 2×2 tables would establish the ability for a reader to differentiate tumoral cells from non-tumoral ones in lung samples with and without tumor, values were obtained for sensitivity, specificity, likelihood ratio (+), likelihood ratio (–), likelihood ratio (+)/likelihood ratio (–), and odds ratio, their confidence intervals, and the value of χ^2.

Table 2/1. CHARACTERISTICS OF 76 CASES OF LUNG CANCER.

Nº	AGE (years)	GENDER	TYPE OF TUMOR	STAGING			PRIMARY TUMOR		TEST	TYPE OF LUNG RESECTION
				T	N	M	Φ MAXIMAL (cm)	AREA (cm^2)		
1	44	FEMALE	SQUAMOUS CELL	2	0	X	3	9	LACTO-ORCEIN	LOBECTOMY
2	57	MALE	SQUAMOUS CELL	3	1	X	10	75	LACTO-ORCEIN	NEUMONECTOMY
3	73	MALE	SQUAMOUS CELL	3	0	X	2.6	6.5	LACTO-ORCEIN	LOBECTOMY
4	72	MALE	ADENOCARCINOMA	4	1	X	4.2	12.6	LACTO-ORCEIN	LOBECTOMY
5	56	MALE	ADENOCARCINOMA	2	0	X	4	14	LACTO-ORCEIN	LOBECTOMY
6	56	MALE	ADENOCARCINOMA	4	0	1	3.5	10.5	LACTO-ORCEIN	LOBECTOMY
7	50	MALE	ADENOCARCINOMA	2	1	X	4	14	LACTO-ORCEIN	LOBECTOMY
8	52	MALE	LARGE-CELL NEURENDOCRINE CANCER	2	0	X	3.5	10.5	LACTO-ORCEIN	LOBECTOMY
9	53	MALE	SQUAMOUS CELL	4	2	X	2	4	LACTO-ORCEIN	LOBECTOMY
10	69	MALE	SQUAMOUS CELL	2	0	X	4	12	LACTO-ORCEIN	NEUMONECTOMY
11	68	MALE	SQUAMOUS CELL	2	0	X	5	20	LACTO-ORCEIN	LOBECTOMY
12	46	MALE	SQUAMOUS CELL	1	2	X	2.2	4.4	LACTO-ORCEIN	NEUMONECTOMY
13	72	MALE	SQUAMOUS CELL	2	0	X	4	10	LACTO-ORCEIN	LOBECTOMY
14	79	MALE	SQUAMOUS CELL	1	0	X	2.5	5	LACTO-ORCEIN	LOBECTOMY
15	68	MALE	ADENOCARCINOMA	2	0	X	3	9	LACTO-ORCEIN	LOBECTOMY
16	62	MALE	LYMPHOMA	4	X	X	3.5	8.4	LACTO-ORCEIN	LOBECTOMY
17	78	MALE	SQUAMOUS CELL	2	0	X	6	36	LACTO-ORCEIN	LOBECTOMY
18	78	MALE	ADENOCARCINOMA	2	0	1	1.5	1.35	LACTO-ORCEIN	ATYPICAL RESECTION
19	62	MALE	SQUAMOUS CELL	3	0	X	9	63	LACTO-ORCEIN	NEUMONECTOMY
20	54	FEMALE	ADENOCARCINOMA	1	0	X	2.5	5	LACTO-ORCEIN	LOBECTOMY
21	71	MALE	SQUAMOUS CELL	2	0	X	4	10	LACTO-ORCEIN	LOBECTOMY
22	76	MALE	LARGE-CELL NEURENDOCRINE CANCER	2	0	X	4.2	10.5	LACTO-ORCEIN	LOBECTOMY
23	79	FEMALE	PLEOMORPHIC SARCOMA	2	0	X	4.5	18.9	LACTO-ORCEIN	LOBECTOMY
24	61	MALE	SQUAMOUS CELL	1	0	X	2.5	6.25	LACTO-ORCEIN	LOBECTOMY
25	73	FEMALE	ADENOCARCINOMA	2	0	X	3.5	10.5	LACTO-ORCEIN+GRAM	NEUMONECTOMY
26	64	MALE	ADENOCARCINOMA	2	1	X	12	144	GRAM	NEUMONECTOMY
27	56	FEMALE	SQUAMOUS CELL	3	1	X	5	17.5	GRAM	LOBECTOMY
28	54	MALE	ADENOCARCINOMA	1	0	X	2.5	5.75	GRAM	LOBECTOMY
29	49	MALE	ADENOCARCINOMA	2	1	X	4	15.6	GRAM	LOBECTOMY
30	57	MALE	SQUAMOUS CELL	3	0	X	5.5	16.5	GRAM	LOBECTOMY
31	78	FEMALE	SQUAMOUS CELL	2	0	X	3	8.4	GRAM	LOBECTOMY
32	72	MALE	SQUAMOUS CELL	2	1	X	6	33	GRAM	LOBECTOMY
33	79	MALE	SQUAMOUS CELL	2	0	0	4	14	GRAM	LOBECTOMY
34	53	MALE	SQUAMOUS CELL	2	0	X	4	12	GRAM	LOBECTOMY
35	66	MALE	LARGE-CELL NEURENDOCRINE CANCER	2	1	X	2	3.6	GRAM	LOBECTOMY
36	73	MALE	SQUAMOUS CELL	3	0	X	7	35	GRAM	LOBECTOMY
37	74	MALE	SQUAMOUS CELL	2	0	X	3.5	8.75	GRAM	NEUMONECTOMY
38	58	MALE	SQUAMOUS CELL	3	0	X	4.5	15.8	GRAM	ATYPICAL RESECTION
39	49	FEMALE	LARGE-CELL NEURENDOCRINE CANCER	3	0	0	4	14	GRAM	LOBECTOMY
40	71	FEMALE	ADENOCARCINOMA	1	1	0	2.5	5	GRAM	NEUMONECTOMY
41	67	MALE	ADENOCARCINOMA	2	2	0	6	30	GRAM	NEUMONECTOMY

Nº	AGE (years)	GENDER	TYPE OF TUMOR	STAGING			PRIMARY TUMOR		TYPE STAINING	TYPE OF LUNG RESECTION
				T	N	M	Φ MAXIMAL (cm)	AREA (cm^2)		
42	49	MALE	SQUAMOUS CELL	2	1	X	5	20	GRAM	NEUMONECTOMY
43	55	MALE	ADENOCARCINOMA	3	2	X	4.5	11.3	GRAM	LOBECTOMY
44	58	MALE	INFLAMMATORY PSEUDOTUMOR	4	X	X	3	4.5	GRAM	LOBECTOMY
45	74	MALE	PLEOMORPHIC SARCOMA	2	0	0	4	14	GRAM	LOBECTOMY
46	59	MALE	SQUAMOUS CELL	2	0	X	1.5	1.95	GRAM	LOBECTOMY
47	74	MALE	SQUAMOUS CELL	2	0	X	6	27	GRAM	LOBECTOMY
48	58	FEMALE	METASTATIC RENAL ADENOCARCINOMA	2	2	1	5	20	GRAM	LOBECTOMY
49	70	MALE	ADENOCARCINOMA	2	0	X	3	9	GRAM-SYTO PLUS PROPIDIUM IODIDE	NEUMONECTOMY
50	63	MALE	SQUAMOUS CELL	2	0	X	3	9	GRAM-SYTO PLUS PROPIDIUM IODIDE	LOBECTOMY
51	55	MALE	ADENOCARCINOMA	1	0	X	4.5	15.8	GRAM-SYTO PLUS PROPIDIUM IODIDE	LOBECTOMY
52	76	MALE	ADENOCARCINOMA	2	0	X	4	16	GRAM-SYTO PLUS PROPIDIUM IODIDE	LOBECTOMY
53	78	MALE	ADENOCARCINOMA	2	0	X	3.5	12.3	GRAM-SYTO PLUS PROPIDIUM IODIDE	ATYPICAL RESECTION
54	81	MALE	SQUAMOUS CELL	2	0	X	5.4	24.3	GRAM-SYTO PLUS PROPIDIUM IODIDE	ATYPICAL RESECTION
55	58	MALE	ADENOCARCINOMA	1	0	X	2.5	5.5	GRAM-SYTO PLUS PROPIDIUM IODIDE	LOBECTOMY
56	71	MALE	SQUAMOUS CELL	2	0	X	2	4	GRAM-SYTO PLUS PROPIDIUM IODIDE	NEUMONECTOMY
57	52	MALE	ADENOCARCINOMA	2	0	X	4.2	10.5	SYTO PLUS PROPIDIUM IODIDE	LOBECTOMY
58	70	MALE	SQUAMOUS CELL	2	0	X	3.5	10.5	SYTO PLUS PROPIDIUM IODIDE	ATYPICAL RESECTION
59	61	MALE	SQUAMOUS CELL	3	0	X	5	15	SYTO PLUS PROPIDIUM IODIDE	LOBECTOMY
60	78	MALE	SQUAMOUS CELL	2	1	X	5.5	22	SYTO PLUS PROPIDIUM IODIDE	LOBECTOMY
61	62	FEMALE	ADENOCARCINOMA	2	0	X	6	24	SYTO PLUS PROPIDIUM IODIDE	NEUMONECTOMY
62	54	MALE	SQUAMOUS CELL	1	0	X	2.5	5	SYTO PLUS PROPIDIUM IODIDE GENOTYPIC TEST	NEUMONECTOMY
63	51	MALE	ADENOCARCINOMA	3	0	X	3.5	10.2	SYTO PLUS PROPIDIUM IODIDE	LOBECTOMY
64	74	MALE	ADENOCARCINOMA	2	1	X	4	14.8	SYTO PLUS PROPIDIUM IODIDE GENOTYPIC TEST	LOBECTOMY
65	65	MALE	ADENOCARCINOMA	3	1	X	4	14	SYTO PLUS PROPIDIUM IODIDE	LOBECTOMY
66	52	FEMALE	ADENOCARCINOMA	1	2	X	2.5	5	SYTO PLUS PROPIDIUM IODIDE	NEUMONECTOMY
67	77	MALE	SQUAMOUS CELL	2	0	X	4.5	15.8	SYTO PLUS PROPIDIUM IODIDE	LOBECTOMY
68	57	FEMALE	ADENOCARCINOMA	2	2	X	3.1	7.75	SYTO PLUS PROPIDIUM IODIDE	LOBECTOMY
69	64	MALE	METASTATIC GASTRIC ADENOCARCINOMA	3	0	X	8.0	56	GENOTYPIC TEST	LOBECTOMY
70	76	MALE	ADENOCARCINOMA	2	0	1	5.5	22	GENOTYPIC TEST	LOBECTOMY
71	65	FEMALE	METASTATIC ENDOMETRIAL ADENOCARCINOMA	1	0	1	2.8	5.6	GENOTYPIC TESTS	LOBECTOMY
72	56	MALE	SQUAMOUS CELL	1	0	0	2.5	5.5	GENOTYPIC TESTS	NO RESECTION
73	52	FEMALE	ADENOCARCINOMA	2	0	X	3.5	7	GENOTYPIC TESTS	LOBECTOMY
74	52	FEMALE	ADENOCARCINOMA	4	0	0	2.0	4	GENOTYPIC TESTS	NO RESECTION
75	58	MALE	ADENOCARCINOMA	3	0	0	8.0	56	GENOTYPIC TESTS	NEUMONECTOMY
76	71	FEMALE	ADENOCARCINOMA	1	0	0	3.0	9	GENOTYPIC TESTS	LOBECTOMY

Table 2/2. DEMOGRAPHIC AND CLINICAL DATA OF ALL CASES (n = 76).

AGE (years)	MEAN ± 1SD. (MAXIMUM, MINIMUM)	63.9±10.0 (44-81)		
GENDER	FEMALE	16 (21.1%)		
	MALE	60 (78.9%)		
DATE OF SURGERY	2008	20 (26.3%)		
	2009	21 (27.6%)		
	2010	20 (26.3%)		
	2011	7 (9.2%)		
	2012	8 (10.5%)		
TYPE OF TUMOR	ADENOCARCINOMA	31 (40.8%)		
	SQUAMOUS- CELL CANCER	34 (44.7%)		
	PULMONARY B LYMPHOMA	1 (1.3%)		
	METASTATIC CANCER	3 (3.9%)		
	LARGE-CELL NEURENDOCRINE CANCER	4 (5.3%)		
	PLEOMORPHIC SARCOMA	2 (2.6%)		
	INFLAMMATORY PSEUDOTUMOR	1 (1.3%)		
TNM DESCRIPTOR		T	N	M
	0		54 (71.1%)	9 (11.8%)
	1	13 (17.1%)	13 (17.1%)	5 (6.6%)
	2	43 (56.6%)	7 (9.2%)	
	3	14 (18.4%)		
	4	6 (7.9%)		
	X		2 (2.6%)	62 (81.6%)
TEST (n=96)	LACTO-ORCEIN	25 (26.0%)		
	GRAM	31 (32.3%)		
	SYTO PLUS PROPIDIUM IODIDE	20 (20.8%)		
	GENOTYPIC TEST	20 (20.8%)		
TYPE OF LUNG RESECTION	ATYPICAL RESECTION	5 (6.6%)		
	LOBECTOMY	53 (69.7%)		
	NEUMONECTOMY	16 (21.1%)		
	NO RESECTION	2 (2.6%)		

CHAPTER 3

LACTO-ORCEIN STAINING: PRESENCE OF COCCOBACILLI INSIDE MALIGNANT CELLS

1. Introduction

We stained samples with lacto-orcein dye in order to compare characteristics between cells coming from tumor tissue and cells coming from normal neighboring tissue.

2. Materials and methods

Samples from tumoral and normal lung were obtained directly after surgical procedure and were processed within 45 min inside a laminar-flow cabin (model Telstar bio II A/P, Barcelona, Spain) in maximal sterility conditions. Both tissues (tumoral and normal) were mildly pressed on sterilised microscope slides, without lateral displacements to avoid artifacts. Smear samples of the first 25 cases (1-25, Table 3/1) were analyzed with the same procedure used to detect symbionts inside paramecia.[1] In short, cells were placed on a microscope slide, fixed for a few seconds in osmic acid vapor, and treated with a drop of acetone and a drop of lacto-orcein stain. The preparation was then analyzed with a dark-phase-contrast microscope (×2000). A sterilised slide without any sample was stained with dye as a control for every analysis, to rule out precipitations.

Diameters of lung cells (tumoral and normal) were measured using Soft Imaging System (Olympus Co Measure), and classified by size (small ≤ 2.9 µm, intermediate 3.0-11.9 µm, and large ≥12 µm) to calculate the size distribution. Other cell characteristics such as nuclei segmentation, vacuoles, and cell necrosis were recorded.

The presence of rods was investigated: these organisms, with straight, curved, or elliptical shapes were recognized by their sharp border, a length of less than 2 µm, and the presence of at least two per cell. Cocci were identified as homogeneous, dark dots, without any clear contents in the interior of the dot.

Because there were difficulties in assessing and measuring coccobacilli, especially cocci, tumoral and benign cells were photographed. Pictures (×2000) were focused to detect cocci and rods with maximal quality, and every photo was consequently directed to focus on only one cell per microscopic field. Pictures sized 4×3 cm with no identification were randomly mixed, and observers were asked to recognize tumor cells, according to the presence of intracellular coccobacilli. Cells were considered tumoral when there was at least two rods or dots per cell; cells were considered benign when they contained no dots or rods.

Because of difficulties in correctly focusing such small structures, observations were done on each photo (one cell) or on clusters of three photos from the same category: tumor or benign cell (three cells). Photos were analysed by one expert and two inexpert readers. The inexpert readers had no specific biology knowledge; one was a computer technician and the other was an economist. They analyzed clusters of three photos. Both received a brief explanation about diagnostic criteria of cocci and rods and had two trial tests that same day, the analysis was done 24 h after the initial tests. Parameters were correlated with type, size, and staging of lung cancer according to TNM classification.[2] See Chapter 2 for statistical analysis.

[1] Beale Gh, Jurand A, Preer Jr. *Three different types of matekiller (mu) particles in Paramecium Aurelia (syngen 1).* J cel sci 1969; 1: 31–4.

[2] Detterbeck fc, boffa dj, tanouet lt. *The new lung cancer staging system.* Chest 2009; 136: 260–71.

3. Results

Tumor samples and neighboring normal lung samples were taken from the 25 first surgical cases (Table 3/1). They corresponded to: 13 (52.0%) squamous-cells cancer, 8 (32.0%) adenocarcinomas, 2 (8.0%) large-cell neuroendocrine tumors, 1 (4.0%) pleomorphic sarcoma, and 1 (4.0%) primary pulmonary B lymphoma (Table 3/2).

21 (84%) patients were men and 4 (16%) were women, with a median age of 64.4 (1SD 11.0) years (Table 3/2). Squamous-cell and adenocarcinoma cells had larger diameters than did normal cells ($p = 0.000$) and squamous cells also exceeded the size of neuroendocrine tumor cells ($p = 0.029$; Table 3/3). But, the size distribution in 2469 non-tumoral and 2178 neoplastic cells classified as small, intermediate, and large did not differ between normal and tumor cells (Table 3/4). Other cellular characteristics such as nuclei segmentation, vacuoles, and cellular necrosis did not differ between malignant and benign cells (Table 3/4).

Cocci and rods were present in more than 84.9% of cancer cells versus in 13.9% of non-tumoral cells ($p = 0.000$; Table 3/5). The rods' mean length was 0.82 µm (1SD 0.3), but cocci were so small and numerous that they were difficult to count or measure with certainty (Table 3/5). However, cocci completely filled the inside of tumoral cells and appeared as dark, round, opaque dots with orcein staining.

When observing photographs (×2000), an experienced member of our group recognized cocci without hesitation in 110 (70%) of 157 photos from tumoral cells and in 10 (10%) of 101 photos from benign cells; and he was certain of cocci's absence in 5 (3%) of 157 photos from tumoral cells and 77 (76%) of 101 photos from benign cells. There were doubts in 42 (27%) of 157 photos of cancer cells and 14 (14%) of 101 photos of benign cells (Chi square [χ^2] = 155.7, $p = 0.000$).

The frequency of rods was similar in squamous-cell cancer and in adenocarcinomas. More than two rods per cell identified cancer trait with a sensitivity ratio of 96.2% (95% CI 90–99), specificity ratio of 78.9% (95% CI 75–85), positive likelihood ratio of 4.5, and a negative likelihood ratio of 0.05, ($\chi^2 = 135$, $p = 0.000$). More than two rods per cell, compared with one or no rods per cell, had an absolute risk of tumor of 80%. Stratification of the number of rods per cell (low ≤2, medium 3–9, and high ≥10) showed the cancer percentage rose from 6.2% for low, to 66.6% for medium, and to 94.5% for high. More than 10 rods per cell were detected in 108 (47%) of 230 cells, of which 102 (94%) were tumors. Less than two rods per cell were recorded in 80 (35%) of 230 cells, of which only 5 (6%) were neoplasm. There was no significant relation between rods and tumor stage (TNM classification; data not shown).

The presence of coccobacilli inside cells led to recognition of malignancy, but this recognition depended on the number of cells of the same category that the observer was evaluating at a time. When the expert observer analyzed only one cell as unique representation of one category (benign or malign), the sensitivity was 91.0%, the specificity 91.8%, the positive likelihood ratio 11, the negative likelihood ratio 0.009, and odds ratio 116. When clusters of three cells (or three pictures) represented the same category, then the expert distinguished malignant or benign cell nearly 100% of the time (Tables 3/6 and 3/7). Results from both inexpert observers when they read clusters of three cells were similar and could not be explained by random chance ($p = 0.000$; Table 3/7).

4. Discussion

The only clear characteristic differences between cancer and normal cells in these results, apart from size, were the presence of intracellular coccobacilli, which were stained with lacto-orcein after surgical procedure. Lacto-orcein is a very simple staining and can be done in a few seconds. Contrary to paramecia, in which presence of endosymbionts can be detected immediately after staining, intratumoral coccobacilli can only be detected 3–4 h after staining. This long time needed for intratumoral coccobacilli to absorb the lacto-orcein stain can be explained by the low rate of penetration of dye inside the cancerous cells. This technical detail is so important that, if this time gap is not taken into account, coccobacilli cannot be reliably detected.

Another recommendation for a correct observation of cocci and rods is the use of a phase-contrast microscope (×2000). It is very useful to take pictures with a high resolution photographic camera, with careful focus on coccobacilli inside of only one cell among the several observed on microscopic fields. Observations from photos can also be improved if the observer looks at the pictures from a distance of 40–50 cm.

The cells studied were mainly from squamous-cell tumors and adenocarcinomas. In all types of cells, we observed two morphotypes: cocci, very numerous and difficult to obtain measures from, and rods, of a length of 1 µm.

Figures 3/1 to 3/16 are samples of cells from squamous-cell carcinomas full of cocci and rods. Figures 3/17 to 3/32 show coccobacilli inside adenocarcinoma cells. Non-tumoral cells contain strange structures (Figures 3/33 to 3/34) or reticular images (Figures 3/35 and 3/36) or irregular long lines (Figures 3/37 and 3/38). Cocci, when observed inside benign cells, are scarce and irregular; rods are usually present by less than two per cell (Figures 3/39 and 3/40).

Intracellular cocci and rods are strongly related with tumoral traits, and their high numbers must contribute to the hypertrophy and anomalous shape of nuclei in neoplastic cells, and are probably related also to functional alterations of the cells. The number of intracellular rods did not differ with post-surgical TNM staging.

Several reasons could explain why the presence of these cellular coccobacilli is not due to an accidental contamination or artifacts. First, cocci and rods are repeatedly present in the different histopathological types of tumors here studied. Second, the time after surgery is too short for cells to have accumulated such high amount of intracellular coccobacilli. Third, cells were managed very carefully with maximal sterility conditions. Fourth, non-tumor lung cells were manipulated under similar experimental conditions and contained few or no cocci or rods. Fifth, there are not coccobacilli on control dyes. Sixth, the presence of intracellular coccobacilli is highly consistent and can be identified by inexpert people with minimal training.

In fact, of 199 cells (113 from tumors) an expert could recognize coccobacilli and, by consequence, neoplastic cells in 103 of 113 cells ($\chi^2 = 133$, $p = 0.000$; Table 3/6).

With blocks of three cells of the same category, one expert detected 32 of 32 tumors ($\chi^2 = 48$, $p = 0.000$), and two non-expert observers recognized 30 of 35 and 34 of 37 cancers, respectively ($\chi^2 = 19.8$, $p = 0.000$ and $\chi^2 = 22.4$, $p = 0.000$; Table 3/7).

The interpretation can be double, it could be chromosome pulverization or bacteria, and both can occur together.

Chromosome pulverization is a phenomenon that is rarely described in tumors and occurs mainly during viral cellular infection. Chromosome fragmentation is observed better during metaphase.[3] Furthermore, intracellular cocci and rods have constant morphologies and sizes, which would not be expected in chromosome pulverization, and they are present in practically all tumor cells. These observations rule out the hypothesis of chromosome pulverization. Further information on this point will be discussed in the next chapters.

[3] O'Neill Fj, Miles Cp. *Chromosome Changes in Human Cells induced by Herpes Simplex, Types 1 and 2.* Nature 1969; 223: 851–2.

Table 3/1. CHARACTERISTICS OF 25 CASES. LACTO-ORCEIN STAINING.

N°	AGE (years)	GENDER	TYPE OF TUMOR	STAGING			PRIMARY TUMOR		N° RODS PER CELL (mean)	TYPE OF LUNG RESECTION
				T	N	M	Φ MAXIMAL (cm)	AREA (cm^2)		
1	44	FEMALE	SQUAMOUS CELL	2	0	X	3	9	6.25	LOBECTOMY
2	57	MALE	SQUAMOUS CELL	3	1	X	10	75	9.68	NEUMONECTOMY
3	73	MALE	SQUAMOUS CELL	3	0	X	2.6	6.5	8.33	LOBECTOMY
4	72	MALE	ADENOCARCINOMA	4	1	X	4.2	12.6	5	LOBECTOMY
5	56	MALE	ADENOCARCINOMA	2	0	X	4	14	5	LOBECTOMY
6	56	MALE	ADENOCARCINOMA	4	0	1	3.5	10.5	10	LOBECTOMY
7	50	MALE	ADENOCARCINOMA	2	1	X	4	14	9.5	LOBECTOMY
8	52	MALE	LARGE-CELL NEURENDOCRINE CANCER	2	0	X	3.5	10.5		LOBECTOMY
9	53	MALE	SQUAMOUS CELL	4	2	X	2	4	9.16	LOBECTOMY
10	69	MALE	SQUAMOUS CELL	2	0	X	4	12	8.75	NEUMONECTOMY
11	68	MALE	SQUAMOUS CELL	2	0	X	5	20	6.87	LOBECTOMY
12	46	MALE	SQUAMOUS CELL	1	2	X	2.2	4.4	7.5	NEUMONECTOMY
13	72	MALE	SQUAMOUS CELL	2	0	X	4	10	9.5	LOBECTOMY
14	79	MALE	SQUAMOUS CELL	1	0	X	2.5	5	10	LOBECTOMY
15	68	MALE	ADENOCARCINOMA	2	0	X	3	9	10	LOBECTOMY
16	62	MALE	LYMPHOMA	4	X	X	3.5	8.4	8.1	LOBECTOMY
17	78	MALE	SQUAMOUS CELL	2	0	X	6	36	10	LOBECTOMY
18	78	MALE	ADENOCARCINOMA	2	0	1	1.5	1.35	10	ATYPICAL RESECTION
19	62	MALE	SQUAMOUS CELL	3	0	X	9	63		NEUMONECTOMY
20	54	FEMALE	ADENOCARCINOMA	1	0	X	2.5	5	10	LOBECTOMY
21	71	MALE	SQUAMOUS CELL	2	0	X	4	10	10	LOBECTOMY
22	76	MALE	LARGE-CELL NEURENDOCRINE CANCER	2	0	X	4.2	10.5		LOBECTOMY
23	79	FEMALE	PLEOMORPHIC SARCOMA	2	0	X	4.5	18.9		LOBECTOMY
24	61	MALE	SQUAMOUS CELL	1	0	X	2.5	6.25		LOBECTOMY
25	73	FEMALE	ADENOCARCINOMA	2	0	X	3	9		NEUMONECTOMY

Table 3/2. DEMOGRAPHIC AND CLINICAL DATA OF 25 CASES. LACTO-ORCEIN STAINING.

AGE (years)	MEAN ± 1SD. (MAXIMUM, MINIMUM)	64.4 ± 11.0 (44-79)		
MAXIMAL DIAMETER (cm)		3.9 ± 1.9 (2-10)		
AREA (cm^2)		15.5 ± 17.6 (1-75)		
Nº RODS PER CELL		8.6 ± 1.7 (5-10)		
GENDER	FEMALE	4 (16.0%)		
	MALE	21 (84.0%)		
DATE OF SURGERY	2008	20 (80.0%)		
	2009	5 (20.0%)		
TYPE OF TUMOR	ADENOCARCINOMA	8 (32.0%)		
	SQUAMOUS CELL CANCER	13 (52.0%)		
	PULMONARY B LYMPHOMA	1 (4.0%)		
	LARGE-CELL NEURENDOCRINE CANCER	2 (8.0%)		
	PLEOMORPHIC SARCOMA	1 (4.0%)		
TNM DESCRIPTOR		T	N	M
	0		19 (76.0%)	
	1	4 (16.0%)	3 (12.0%)	2 (8.0%)
	2	14 (56.0%)	2 (8.0%)	
	3	3 (12.0%)		
	4	4 (16.0%)		
	X		1 (4.0%)	23 (92.0%)
TYPE OF LUNG RESECTION	ATYPICAL RESECTION	1 (4.0%)		
	LOBECTOMY	19 (76.0%)		
	NEUMONECTOMY	5 (20.0%)		

Table 3/3. TYPES AND DIAMETER OF TUMOR AND BENIGN CELLS.

TYPE	Nº OF CELLS (%)	DIAMETER ($\tilde{X}$ ±1SD) µm	MULTIPLE COMPARISONS P VALUE
ADENOCARCINOMA (A)	83 (24.7%)	17.4 ± 4.4	A vs B P = 0.000 A vs N P = 0. 029
SQUAMOUS CELL CANCER (SQ)	164 (48.8%)	16.7 ± 5.2	SQ vs B P = 0.000
LARGE-CELL NEURENDOCRINE CANCER (N)	8 (2.4%)	12.1 ± 3.5	B vs A P = 0.000 B vs SQ P = 0.000
PRIMARY B LYMPHOMA (L)	20 (6.0%)	14.7 ± 3.8	
BENIGN LUNG CELLS (B)	61 (18.2%)	13.2 ± 4.5	

Table 3/4. SIZE DISTRIBUTION AND CHARACTERISTICS OF CANCER (16 SAMPLES: 2178 CELLS) AND NON TUMORAL CELLS (20 SAMPLES: 2469 CELLS). OSMIUM-LACTO-ORCEIN STAINING (×2000).

SIZE AND CELLS CHARACTERISTICS	PERCENTAGE OF CELLS				STATISTICAL SIGNIFICANCE
	BENIGN		TUMOR		
	MEAN	STANDARD DEVIATION	MEAN	STANDARD DEVIATION	
≤ 2.9 µm	5.9	5.6	9.1	7.5	NON SIGNIFICANT DIFFERENCES
3 -11.9 µm	49.0	24.2	39.1	17.9	
≥ 12 µm	45.1	25.0	51.8	20.0	
NUCLEUS SEGMENTATION	18.0	19.4	14.5	19.8	
VACUOLES	5.4	10.3	1.1	2.1	
CELLULAR NECROSIS	1.0	2.9	3.4	6.4	

Table 3/5. INTRACELLULAR COCCOBACILLI. OSMIUM-LACTO-ORCEIN STAINING (×2.000), PHASE CONTRAST. EXTRACELLULAR COCCOBACILLI, AFTER ITS EXTRUSION FROM CELLS. OSMIUM-LACTO-ORCEIN (L-O) AND GRAM (G) STAINING (×2000). SQUARE AREA = 4 cm^2.

INTRA-CELLULAR COCCI AND RODS		TUMOR	NORMAL LUNG	STATISTICAL SIGNIFICANCE
	Nº CELLS	113	86	
OSMIUM-LACTO-ORCEIN STAINING (L-O)	Nº (%) RODS-BEARING CELLS	103 (91.1)	7 (8.1)	$\chi^2 = 136$ P = 0.000 OR = 116 (38 to 371)
(L-O)	Nº (%) COCCO-BEARING CELLS	96 (84.9)	12 (13.9)	$\chi^2 = 88.7$ P = 0.000 OR = 31 (12 to 73)
(L-O)	RODS LENGTH ($\tilde{X} \pm 1SD$) µm	0.82±0.3*		
EXTRACELLULAR COCCOBACILLI		TUMOR	NORMAL LUNG	STATISTICAL SIGNIFICANCE
(L-O)	Nº SQUARES STUDIED	30	30	
(L-O)	Nº COCCOBACILLI / SQUARE ($\tilde{X} \pm 1SD$)	21.7 ± 8.1	9.07 ± 8.8	T = 5.9 P = 0.000
(L-O)	RODS LENGTH ($\tilde{X} \pm 1SD$) µm	0.69 ± 0.2**	–	
GRAM STAINING (G)		TUMOR	NORMAL LUNG	STATISTICAL SIGNIFICANCE
(G)	COCCI DIAMETER ($\tilde{X} \pm 1SD$) µm	0.35 ± 0.08***	–	* vs ** NSD * vs *** P < 0.05 ** vs *** P < 0.005

Table 3/6. EXPERT READING INTRACELLULAR COCCOBACILLI: ONE CELL REPRESENTS ONLY ONE CATEGORY: TUMOR OR BENIGN CELL, OSMIUM-LACTO-ORCEIN STAINING. PHASE CONTRAST (×2000).

OSMIUM-LACTO-ORCEIN STAINING	TUMORAL CELLS Nº	BENIGN CELLS Nº	TOTAL
COCCOBACILLI +	103	7	110
COCCOBACILLI -	10	79	89
TOTAL	**113**	**86**	**199**

Sensitivity: *91.0 % 95% CI (86%-96%)*
Specificity: *91.8 % 95% CI (86%-96%)*
Likelihood ratio (+): *11*
Likelihood ratio (-): *0.009*
Likelihood ratio (+) / Likelihood ratio (-): *122*
OR: *116 95% CI (32 to 378)*
χ^2 ***(correction Yates):*** *133 (P = 0.000)*

Table 3/7. EXPERT AND INEXPERTS READING INTRACELLULAR COCCOBACILLI: BLOCK OF 3 CELLS ARE FROM THE SAME CATEGORY (TUMOR OR BENIGN CELLS). OSMIUM-LACTO-ORCEIN STAINING. PHASE CONTRAST (×2000).

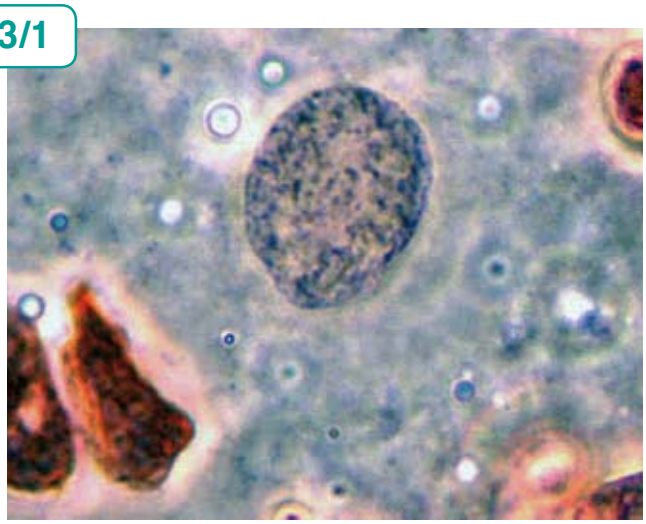
READERS	LACTO-ORCEIN STAINING	Nº BLOCKS WITH THREE CELLS		
		TUMOR	BENIGN	TOTAL
EXPERT	COCCOBACILLI +	32	2	**34**
	COCCOBACILLI -	0	23	**23**
	TOTAL	**32**	**25**	**57**
INEXPERT 1	COCCOBACILLI +	30	6	**36**
	COCCOBACILLI -	5	18	**23**
	TOTAL	**35**	**24**	**59**
INEXPERT 2	COCCOBACILLI +	34	7	**41**
	COCCOBACILLI -	4	18	**22**
	TOTAL	**37**	**25**	**62**

	COMPARISON: EXPERT vs INEXPERT		
	EXPERT	INEXPERT 1	INEXPERT 2
SENSITIVITY (%) 95% CI	100 92 to 100	85 62 to 95	89 80 to 98
SPECIFICITY (%) 95% CI	92 85 to 98	75 60 to 85	75 60 to 85
χ^2 P VALUE	48 0.000	19.8 0.000	22.4 0.000

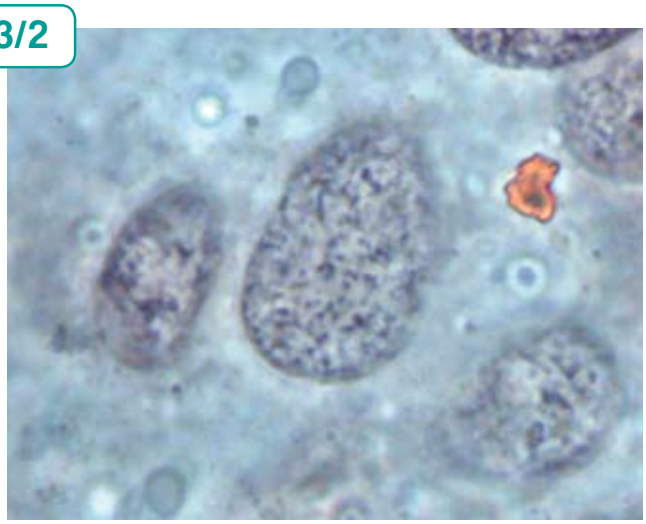
Sqamous-cell cancer. Lacto-orcein staining (×2000)

(FIGURES **3/1** TO **3/16**)

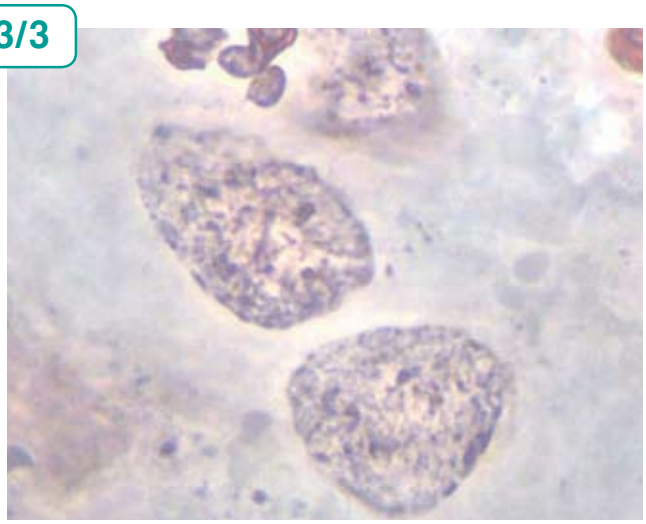
3/1



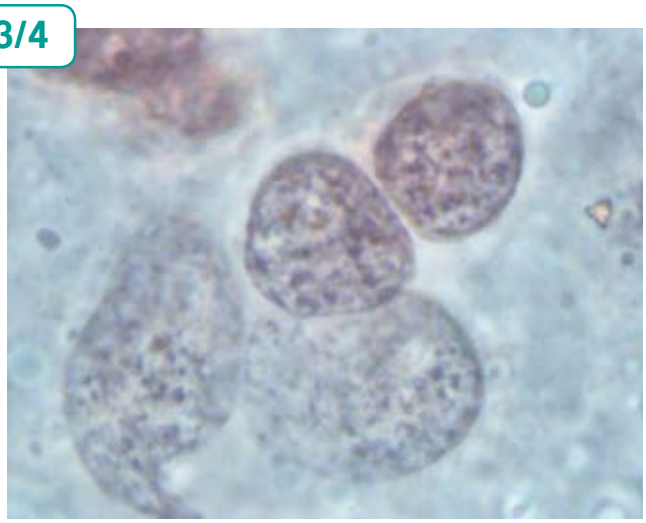
3/2



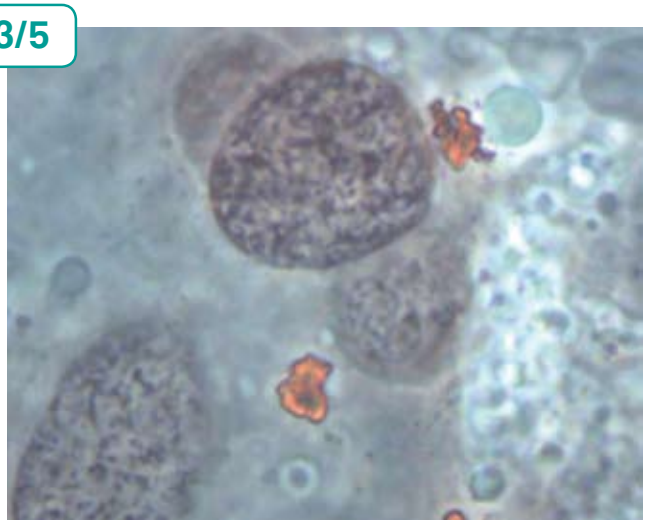
3/3



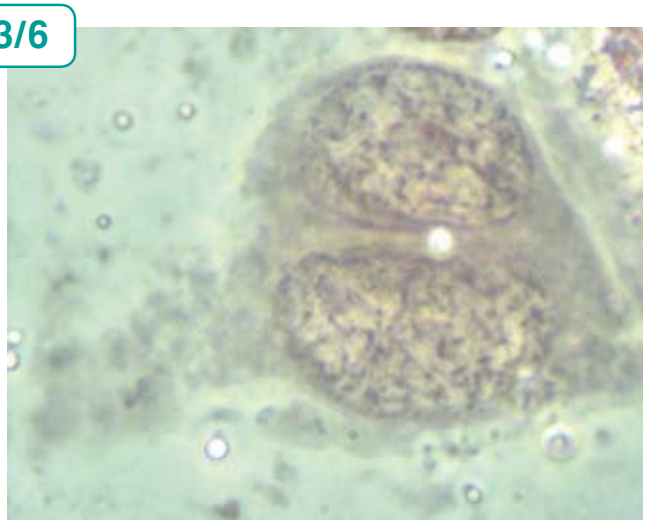
3/4



3/5



3/6



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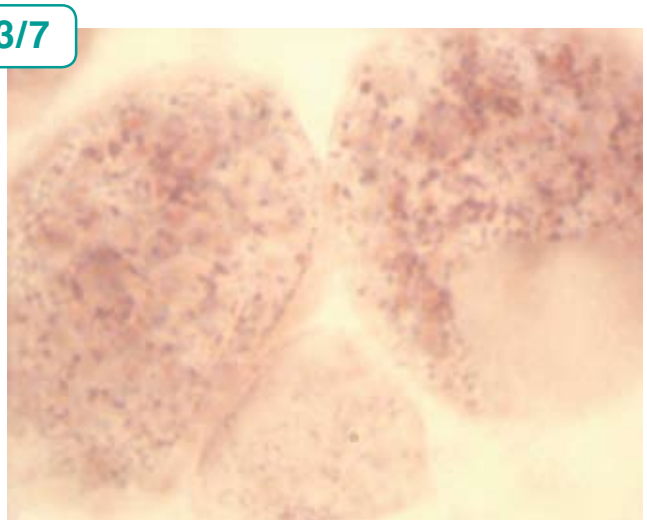

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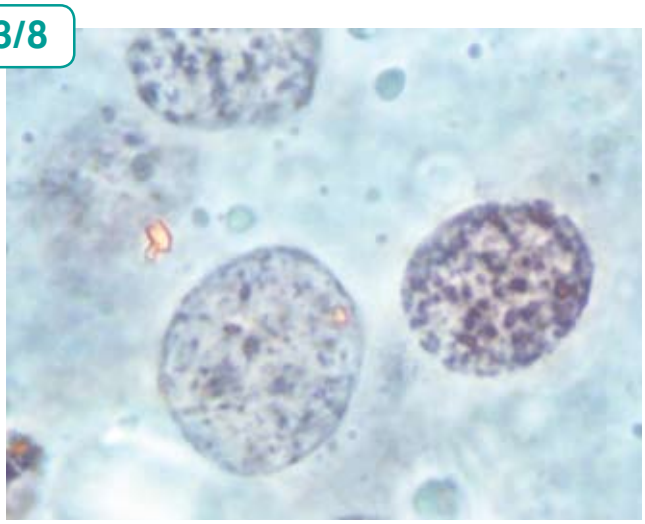

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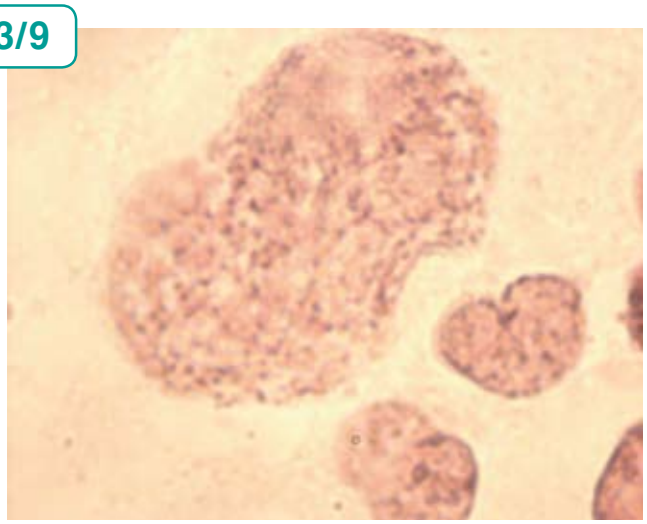

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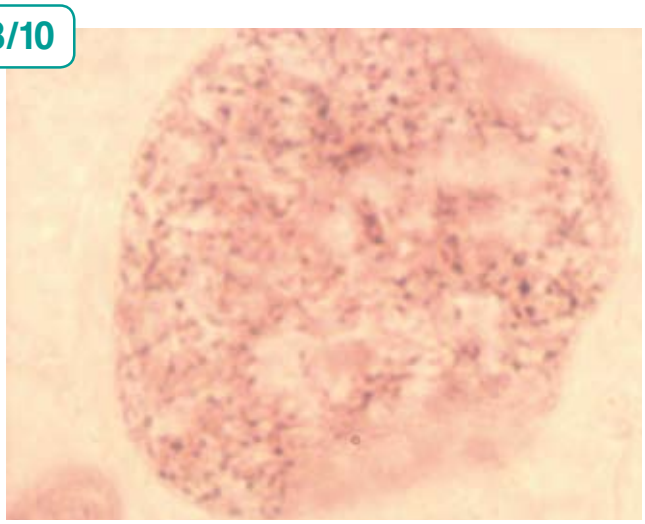

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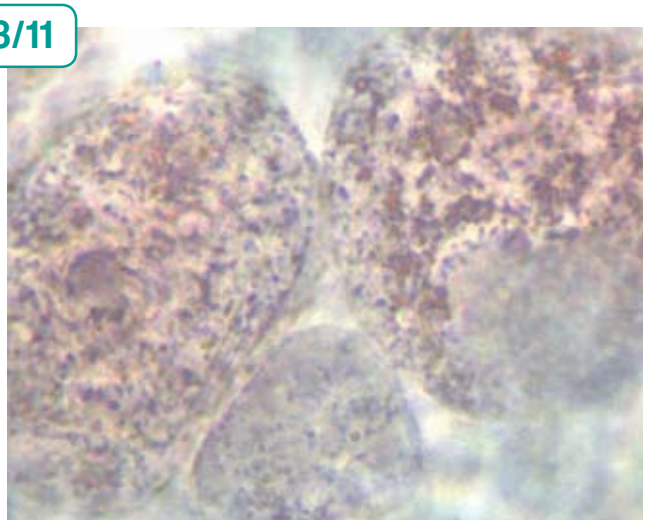

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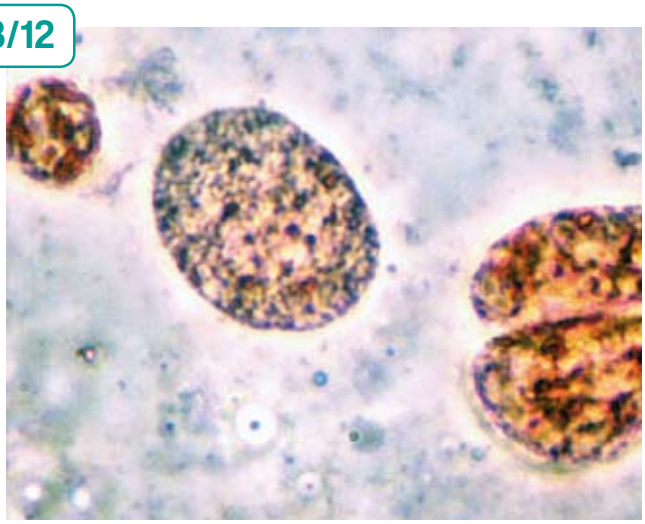

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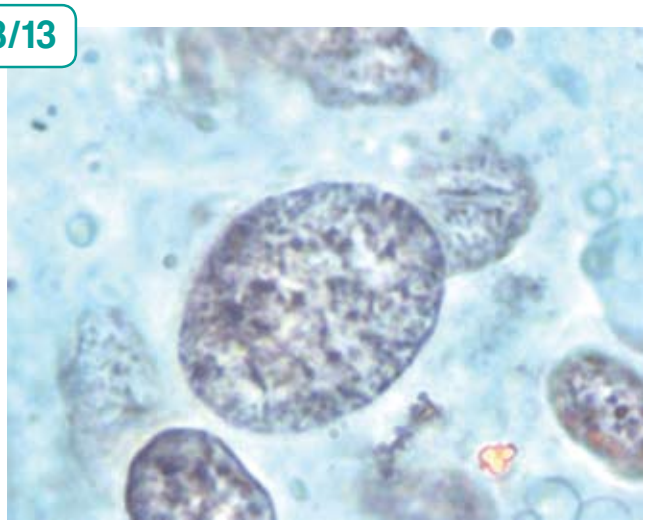

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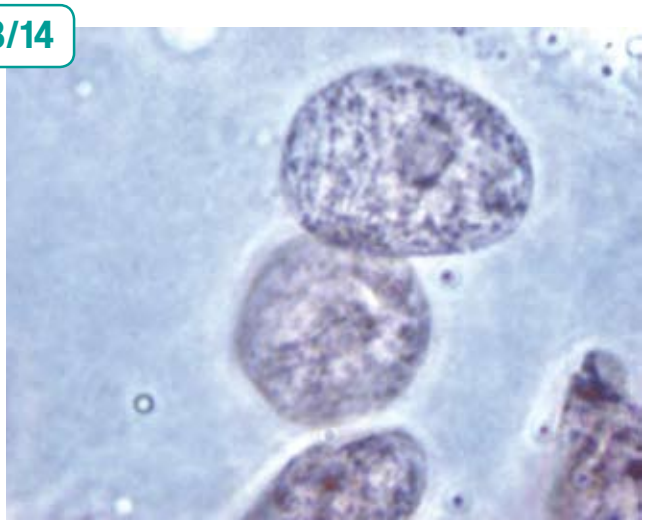

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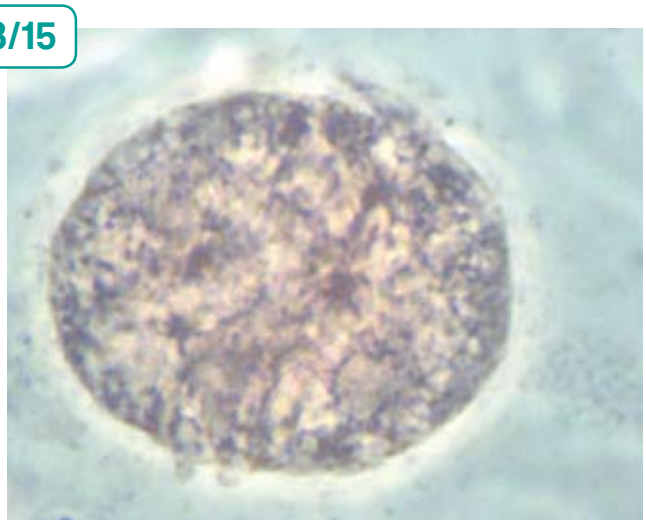

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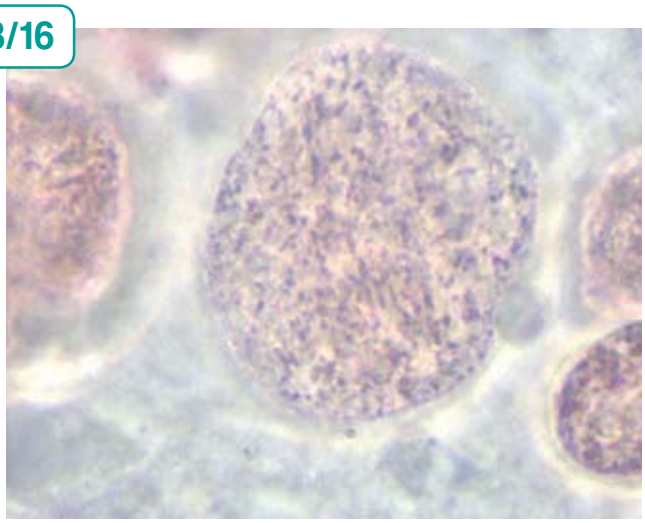
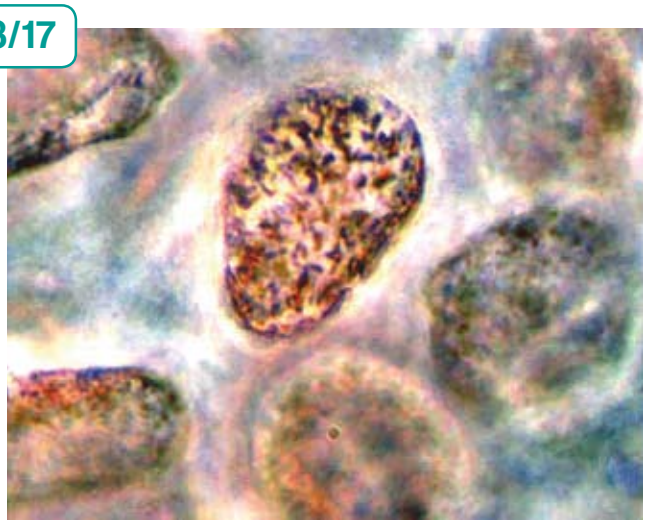


Adenocarcinomas. Lacto-orcein staining (×2000)

(FIGURES 3/17 TO 3/32)

3/17



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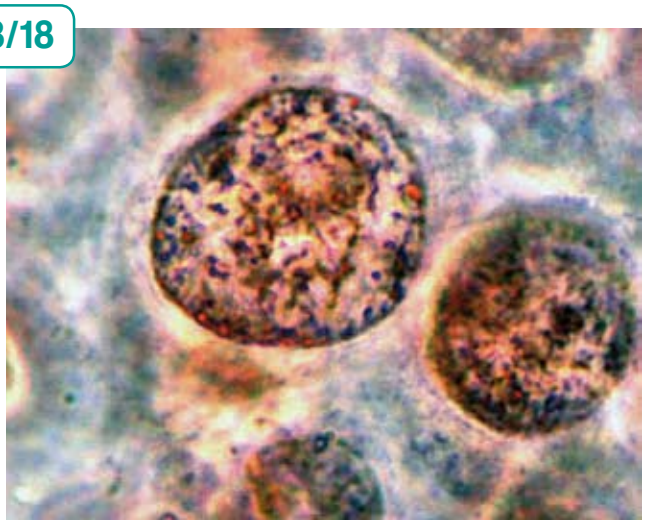

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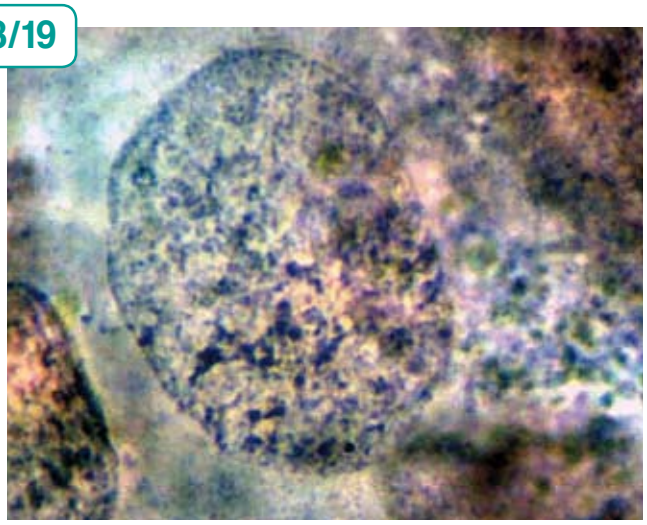

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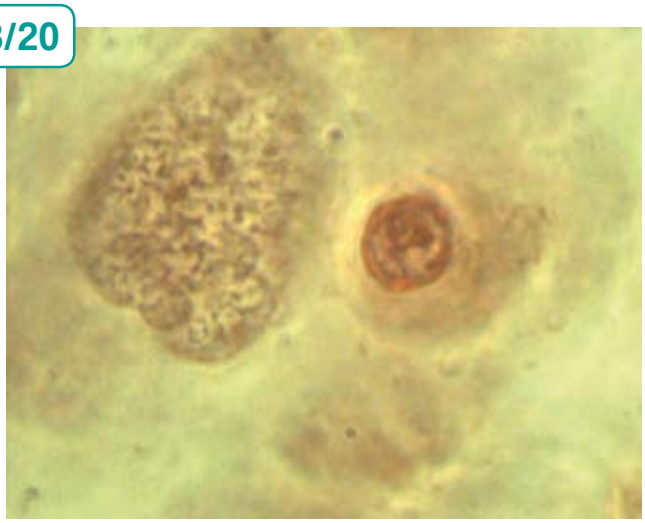

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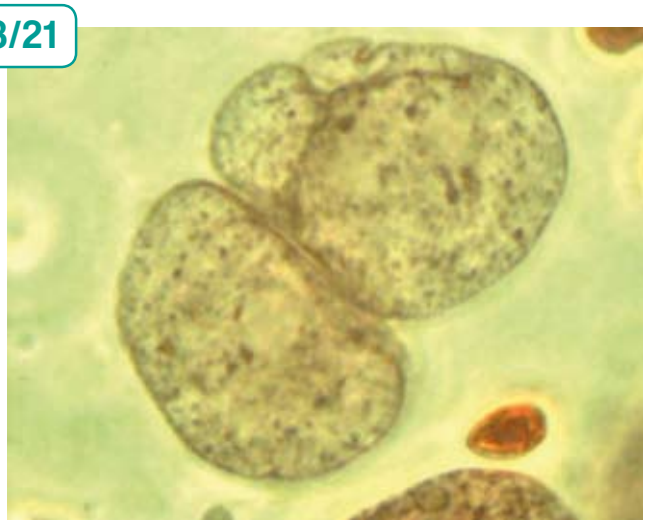

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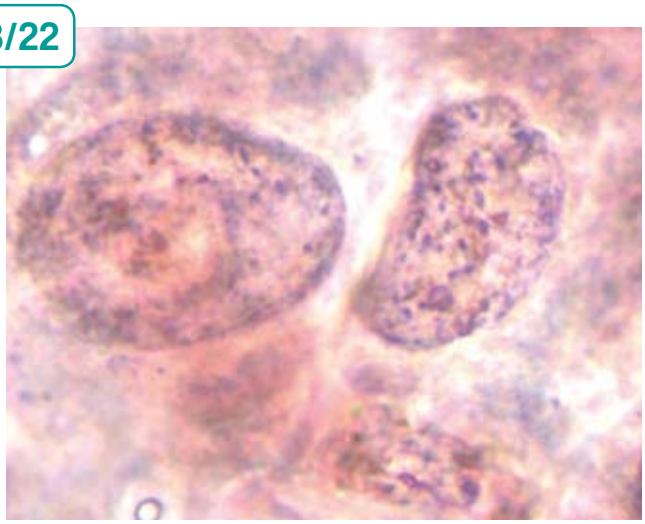

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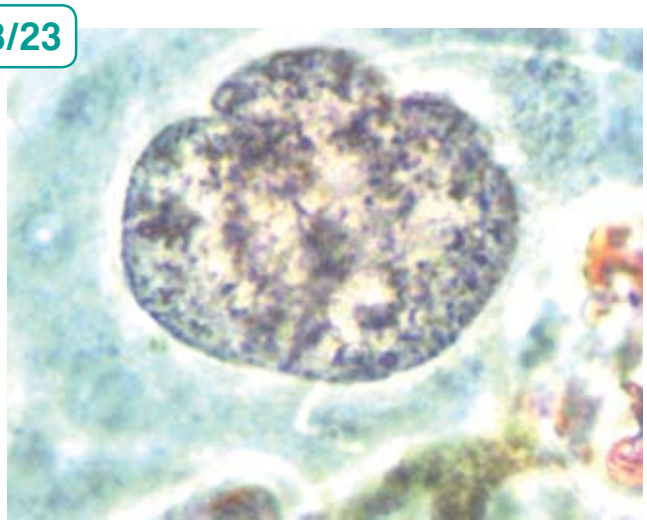

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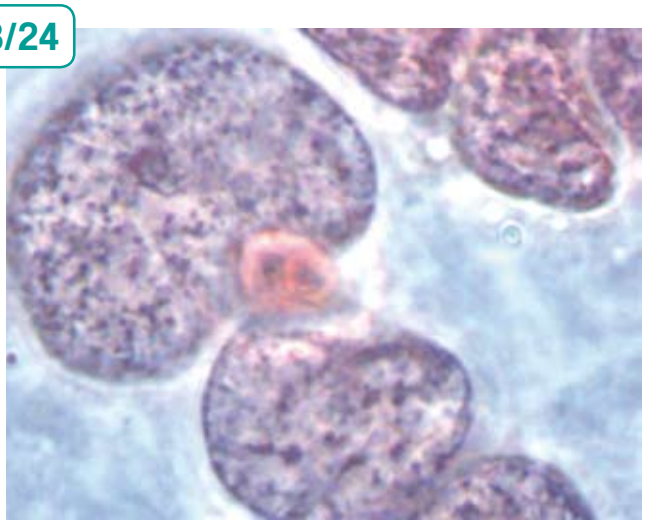

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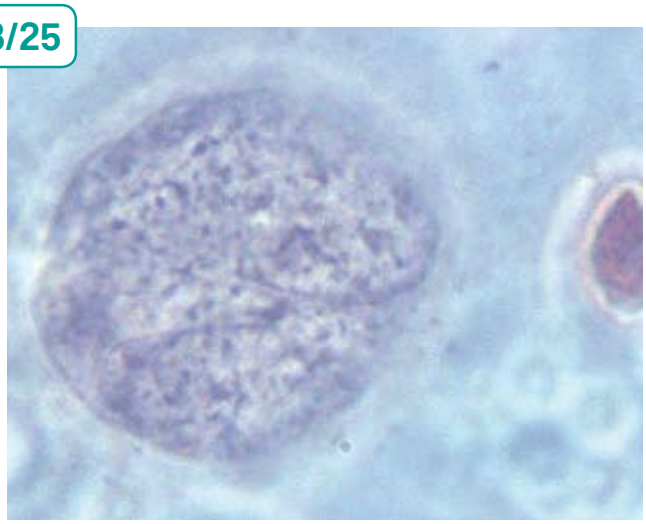

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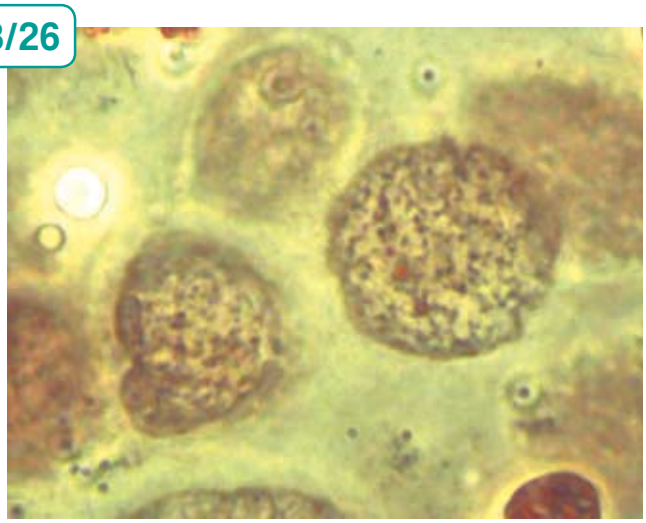

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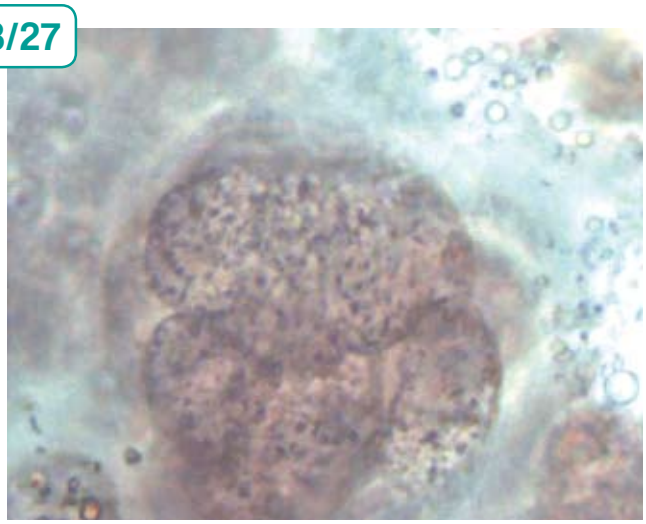

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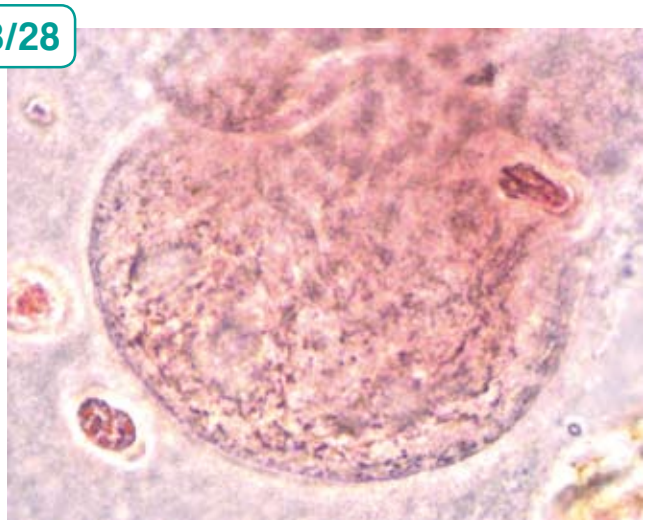

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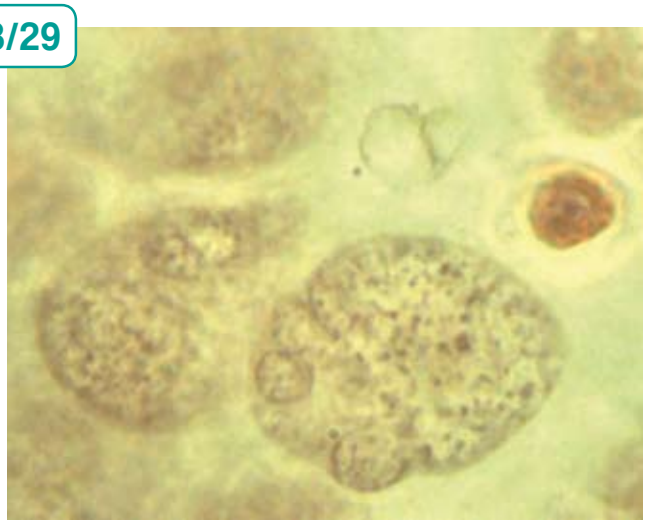

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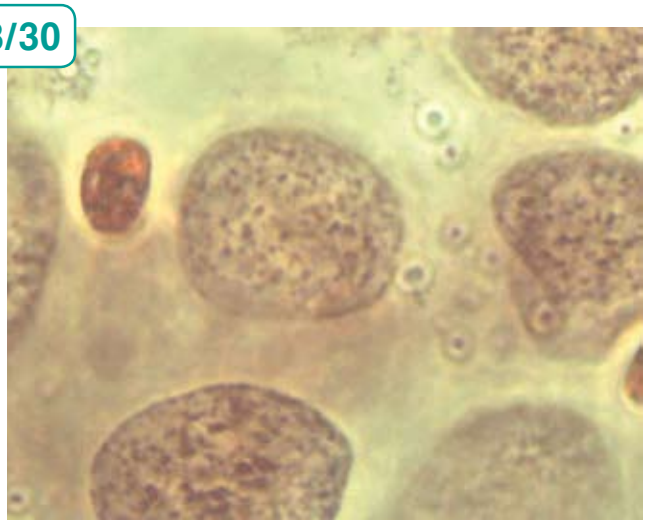

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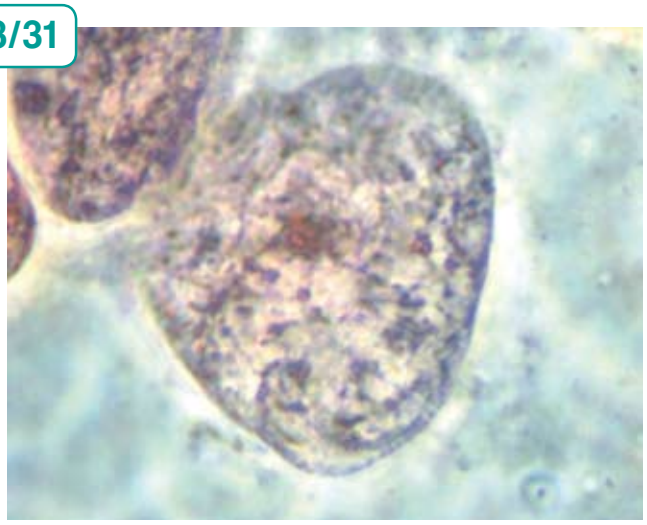

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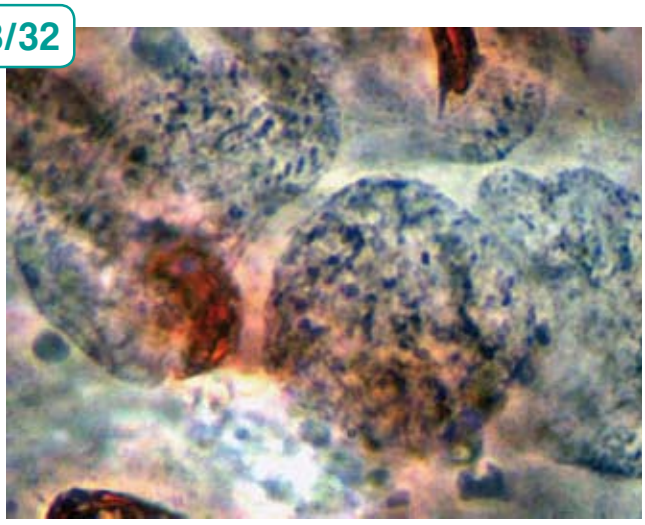
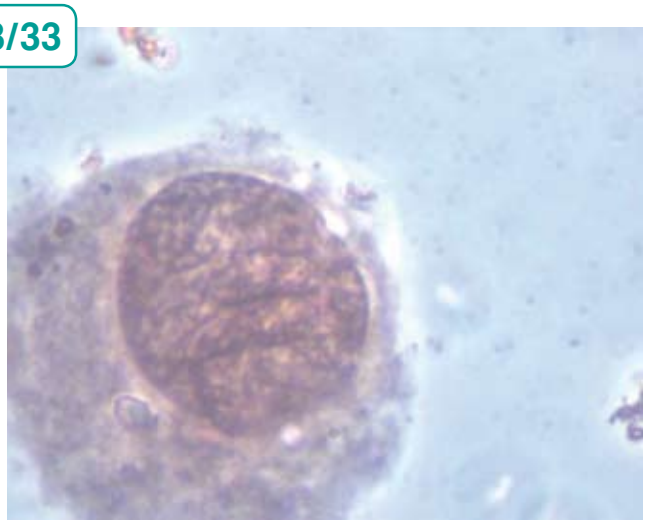


Healthy lung cell. Lacto-orcein staining (×2000)

(FIGURES 3/33 TO 3/40)

3/33



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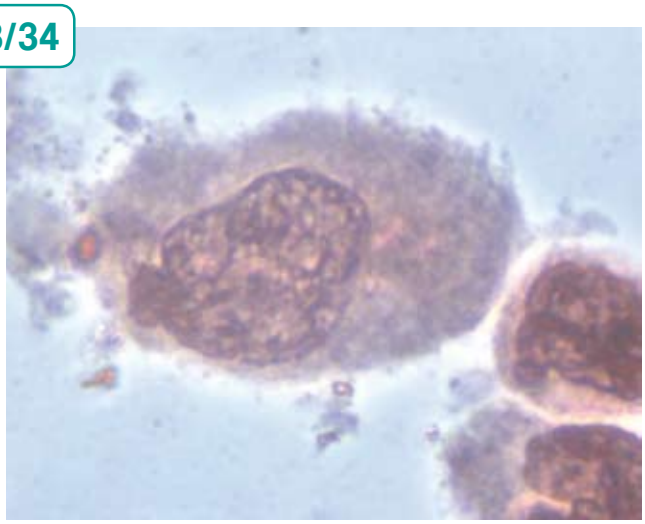

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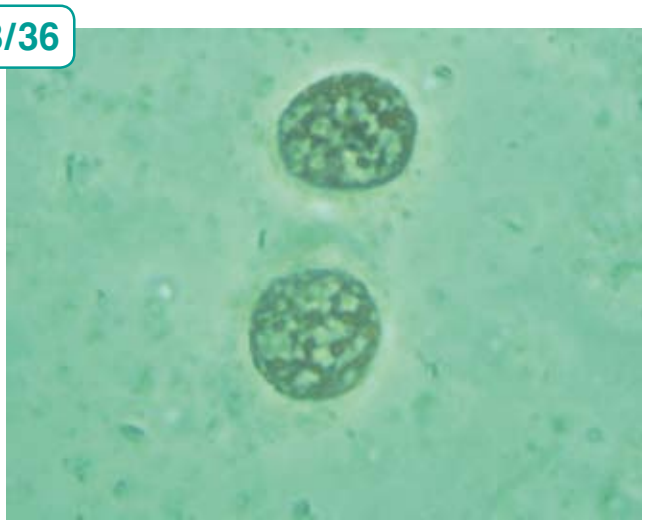

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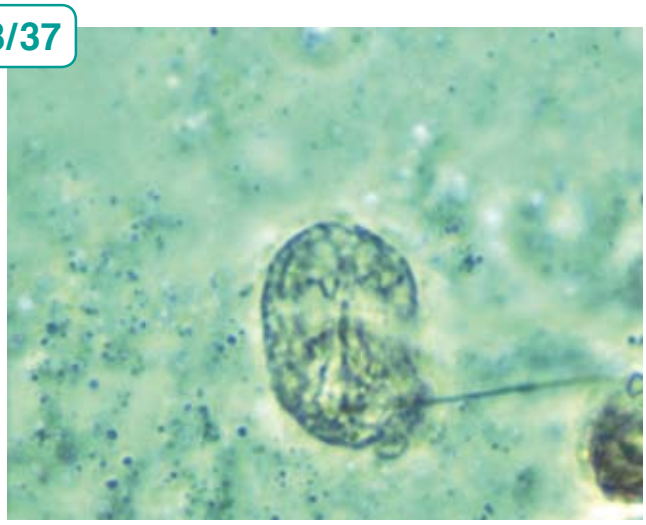

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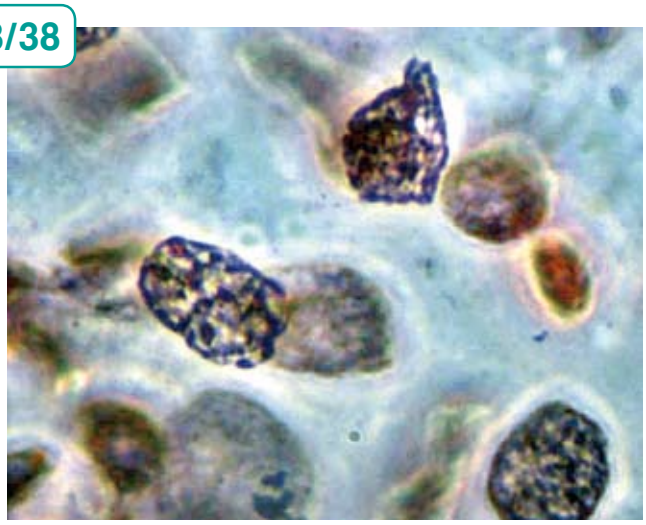

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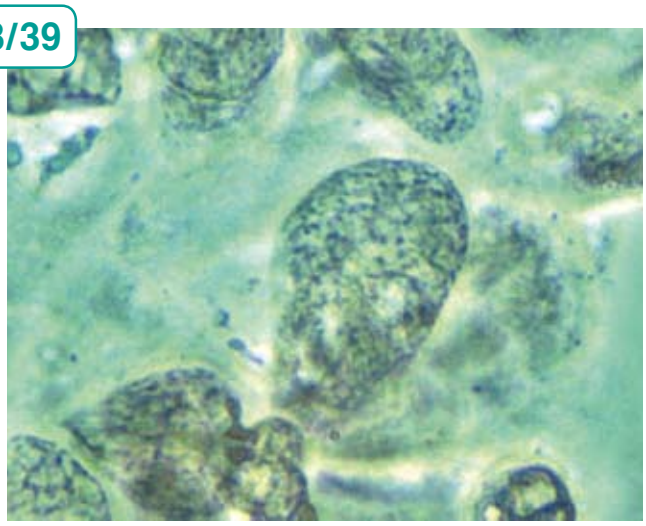

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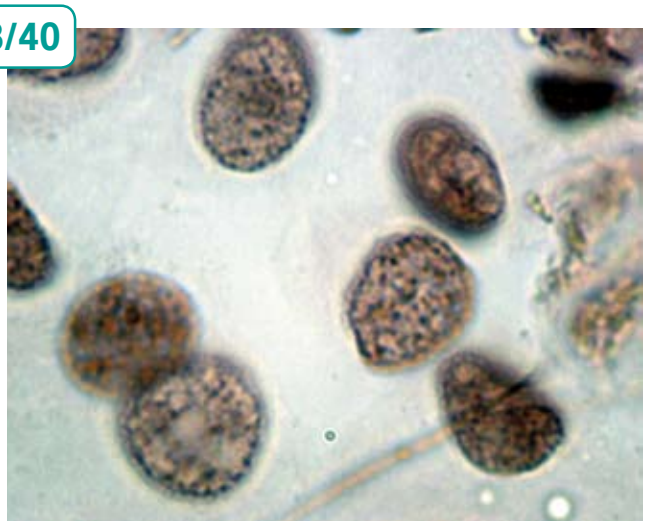


CHAPTER 4

LACTO-ORCEIN STAINING: THE MITOTIC CYCLE

1. Introduction

Orcein dye, or aceto-orcein, has widely been used for the study of cellular mitotic cycle. Lacto-orcein is a chemically close product, in which the acetic acid (or ethanoic acid) is substituted by lactic acid (2-hydroxypropanoic). Lacto-orcein staining permits the observation of clear images of different stages of mitosis, in a similar manner as does aceto-orcein.

2. Materials and methods

We studied ten successive cases of lung cancer and the neighboring non-neoplastic lung tissue, obtained from the same surgical procedures, which we processed using the same methods as described in Chapter 3. Five of these cases corresponded to squamous-cell carcinomas and the remaining 5 were adenocarcinomas. The smears were analysed to identify the different mitotic phases in tumor and benign cells. The cells were classified by mitotic stages (prophase, metaphase, anaphase, telophase, and interphase or resting period) following described morphological criteria. Briefly, prophase is the phase in which chromosomes are condensed as long rod-like structures. Metaphase is the step during which the chromosomes become aligned on the central plate. Anaphase begins with the abrupt separation of the chromatides into daughter chromosomes as they proceed toward opposite poles of cell. Finally, telophase is the phase in which the nuclear membrane becomes reconstituted.[1] Diameters of cells at rest or during mitosis division were measured with the methods described in Chapter 3. For statistical analysis see Chapter 2.

3. Results

1223 benign cells and 2258 cancer cells were analysed. No division phase was observed among benign cells, whereas 62 cancer cells (2.7%) were observed in mitosis (any stage). There were more cells in mitosis in adenocarcinoma samples than in squamous-cell tumor ($p = 0.000$). Lacto-orcein staining did not show coccobacilli inside cells during the mitotic cycle, but these were clearly seen in neoplastic cells at rest.

The cell diameters were randomly measured for 61 benign cells, 22 tumor cells at any mitotic stage, and 253 tumor cells at rest. Maximum diameters were ($\tilde{x}$ [1SD] 13.2 ± 4.5) µm for benign cells, $15.4 \pm (4.8)$ µm for mitotic tumor cells, and $16.8 \pm (4.9)$ µm for tumor cells in interphase (Table 4/1). Diameters of benign cells differed significantly from those of tumor cells in interphase, but not from those of cells in mitotic stages; diameters of tumor cells did not differ between cells in mitotic stages and those in interphase (Table 4/1).

4. Discussion

Although the intuitive idea is that most tumor cells are in active division, only about 3% of all neoplastic cells were reported in mitotic stage with this stain. However, mitosis was more frequently noted in tumor cells than in benign cells, in which none of the more than 1000 cells studied were in mitosis. It is interesting to note that the proportion of cells in mitosis in tumor samples was substantially higher when samples were colored with other techniques (see Chapter 8).

[1] Miles Cp. *The chromosomes and the mitotic cycle.* In: Koss LG, ed. Diagnostics cytology and its histopathological bases. Phyladelphia: JB Lippincott Co, 1979: 99-127.

The distribution of mitotic cells was irregular, most of them were found in clusters and some fields of view had a poor number or no mitotic cells at all. Such an uneven distribution makes the measurement difficult, but the high number of cells studied here make this data more reliable. Moreover, the time spent between surgical procedure and the staining of the samples was within 45 min; therefore, tissue vitality was sufficiently preserved for the sample to express in-vivo events (Figures 4/1 to 4/46).

Why mitosis was more frequent in adenocarcinoma samples than in squamous-cell cancer samples is unclear. Bipolar cell mitosis was the most commonly observed (Figures 4/34 to 4/39), but multipolar cell divisions, in which a cell is capable of multiply into several daughter cells, were also detected (Figures 4/40, 4/42, and 4/43 to 4/45). This multiple cellular division will be further studied in Chapter 8.

What is the relation between intracellular coccobacilli and the steps of mitosis? Coccobacilli were not seen with this dye in cells that were in different stages of mitosis, but they were noted in neighboring tumor cells in interphase (Figure 4/46).

They were not seen during metaphase and, therefore, the presence of these structures cannot be explained by chromosome pulverisation since chromosome pulverisation is commonly seen during metaphase, in which multiple and extreme chromosome damage occurs, with several chromosome fragmentations and frequent exchange configurations. Chromosome fragmentation with multiple secondary constrictions produces various small particles of different forms and sizes. This is a very different picture from the homogeneous aspect of coccobacilli observed in neoplastic cells. Chromosome fragmentation is frequently noted during infection with herpes simplex virus type 2 in normal human cells, and has been observed only occasionally in Burkitt lymphoma.[2] Another argument against the observed structures being the result of chromosome fragmentation is practically their great number inside all interphase cells, and the constant and regular morphology of these structures.[3] Other findings that rule out the cocci and rods structures being a consequence of anomalous chromosomes will be described in Chapter 5.

Table 4/1. DIAMETERS FROM BENIGN AND TUMORAL CELLS DURING MITOSIS OR INTERPHASE (RESTING) STEPS. OSMIUM-LACTO-ORCEIN STAINING (×2000).

		CELLS NUMBER	DIAMETER ($\tilde{X} \pm 1SD$) µm	STATISTICAL SIGNIFICANCE
TUMOR	MITOSIS (1)	22	15.4 ± 4.8	2 vs 3 P = 0.000
	INTERPHASE (2)	253	16.8 ± 4.9	
BENIGN CELLS (3)		61	13.2 ± 4.5	

[2] Miles Cp. *Chromosomes in cancer.* In: Koss LG, ed. Diagnostics cytology and its histopathological bases. Phyladelphia: JB Lippincott Co, 1979: 128-154.

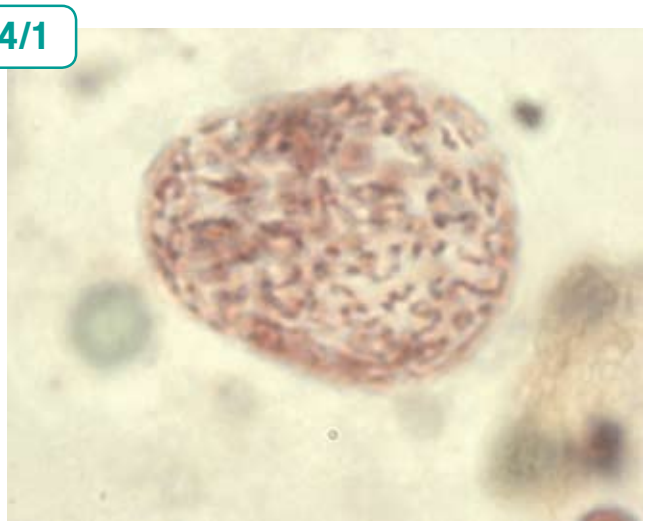
[3] O'Neill Fj, Miles Cp. *Chromosome Changes in Human Cells induced by Herpes Simplex, Types 1 and 2.* Nature 1969; 223: 851–52.

Tumor cell. Lacto-orcein staining (×2000)

FIGURES **4/1** TO **4/46**

→ **Early stage of prophase** (Figures **4/1** to **4/6**)

4/1



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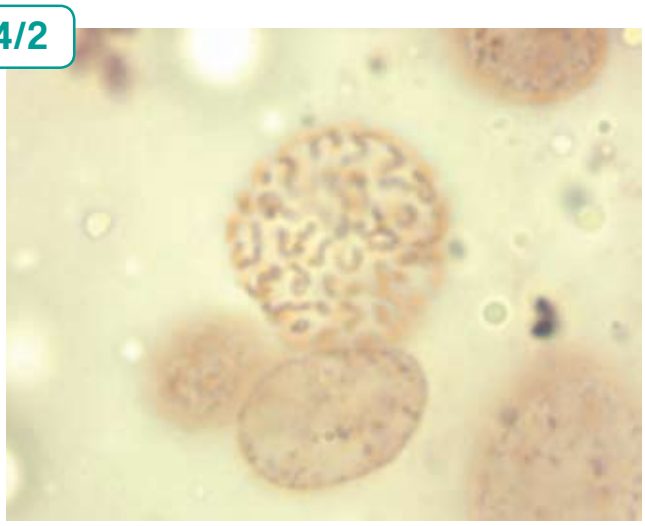

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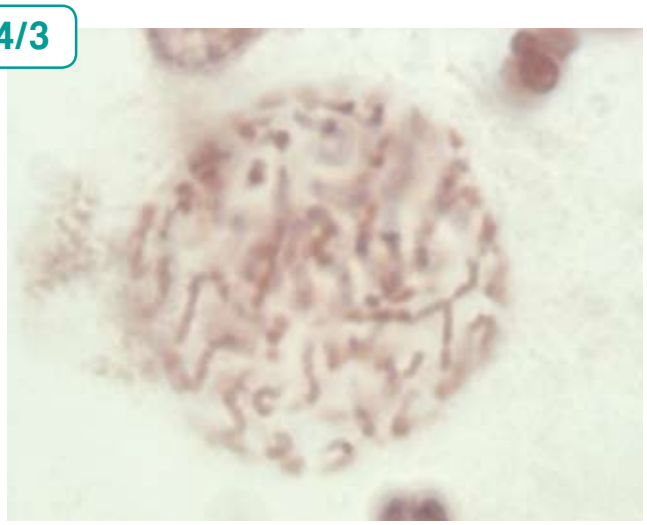

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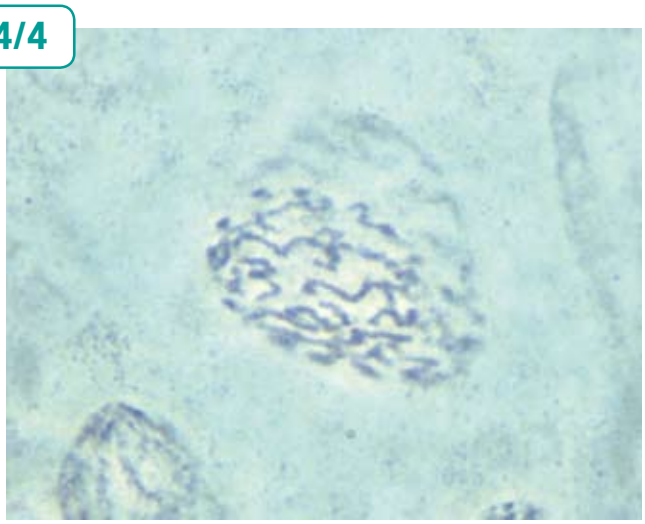

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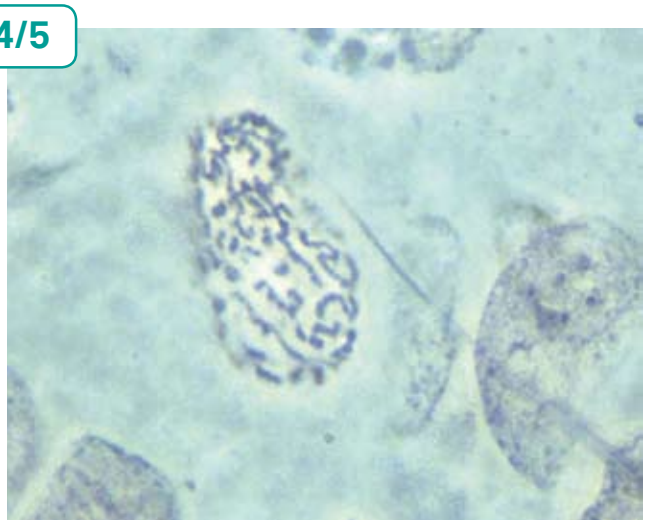

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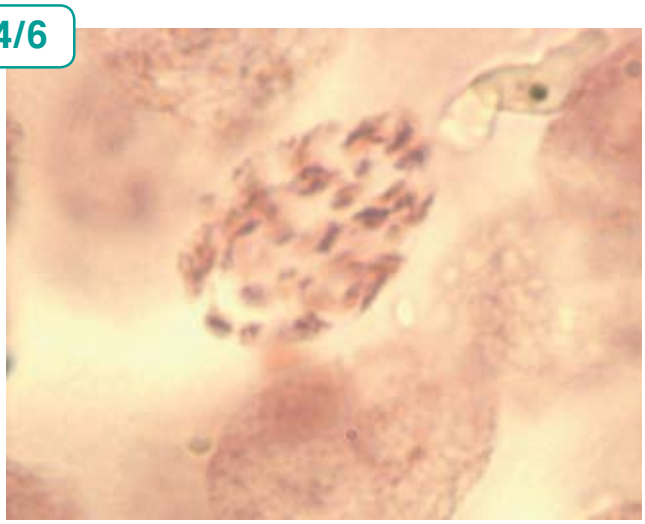
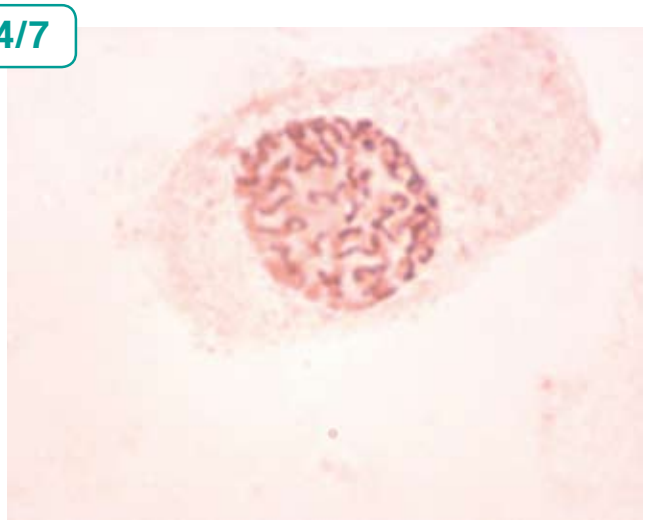


➔ **Late prophase.** Double structure can be visualized in some of these chromosomes (Figures **4/7** to **4/10**)

4/7



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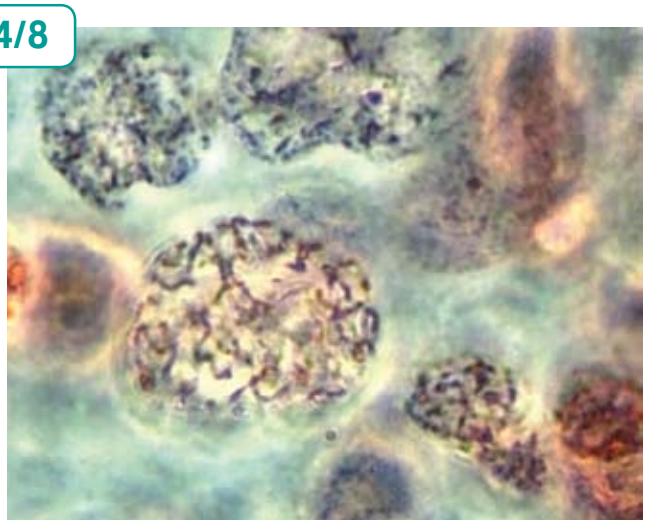

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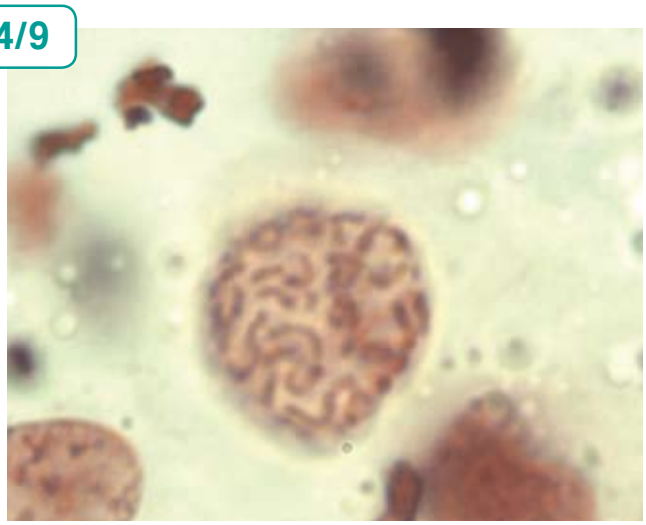

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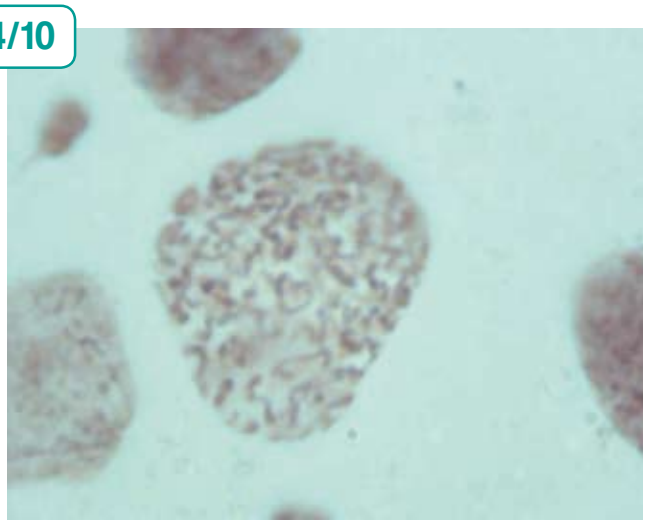
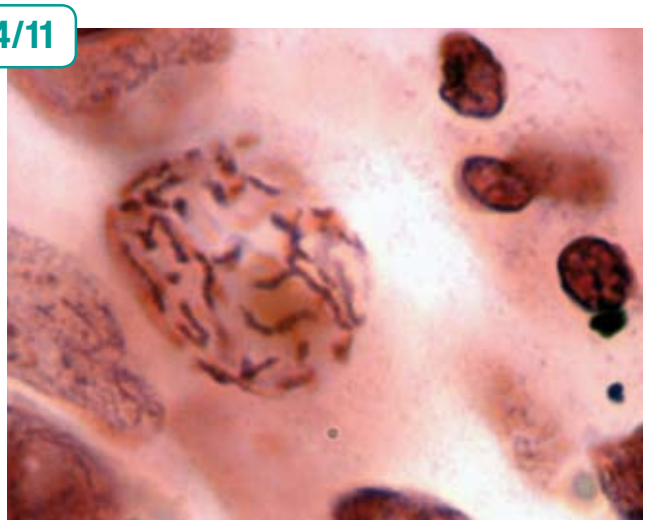


➔ **Late prophase.** Earling metaphase the chromosomes show further contraction (Figures **4/11** to **4/13**)

4/11



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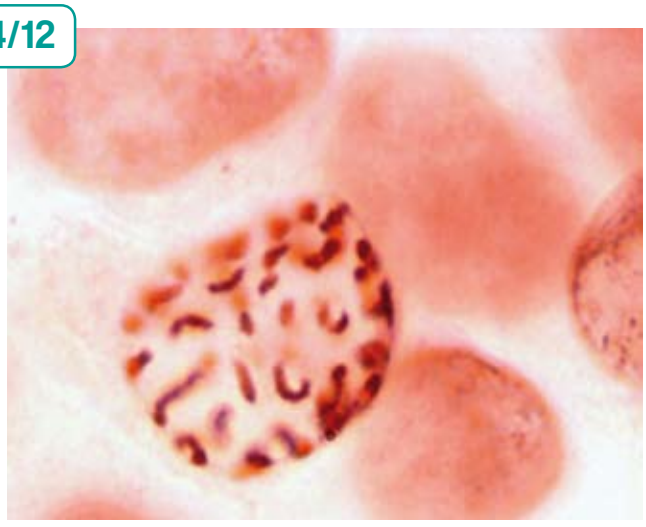

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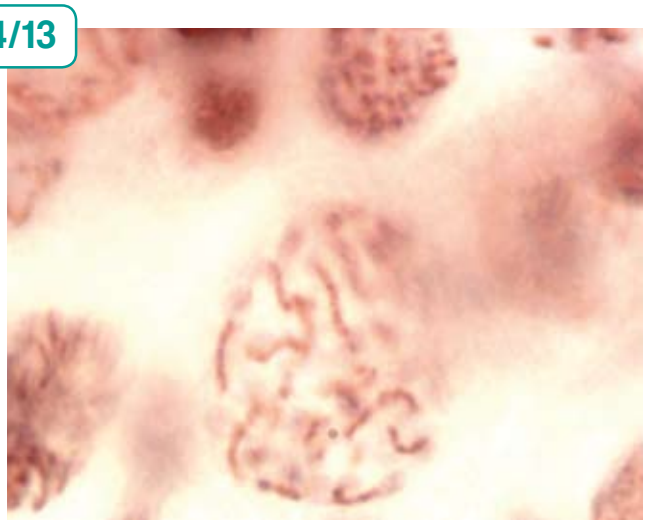
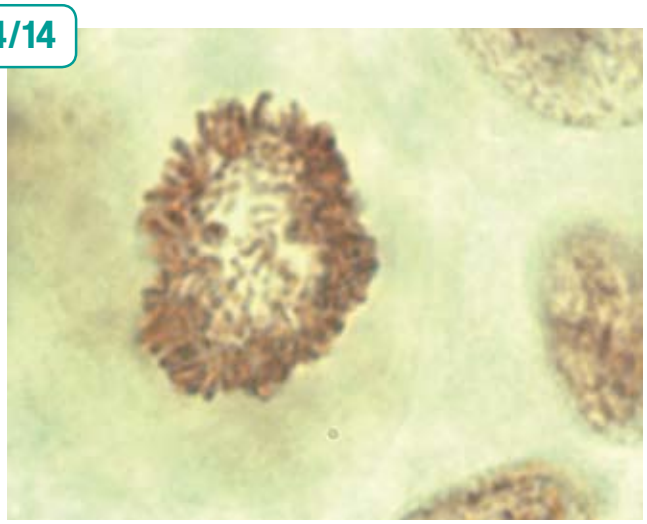


→ **Metaphase:** chromosomes tend to congregated forward the periphery "hollow spindle" (Figures **4/14** to **4/18**)

4/14



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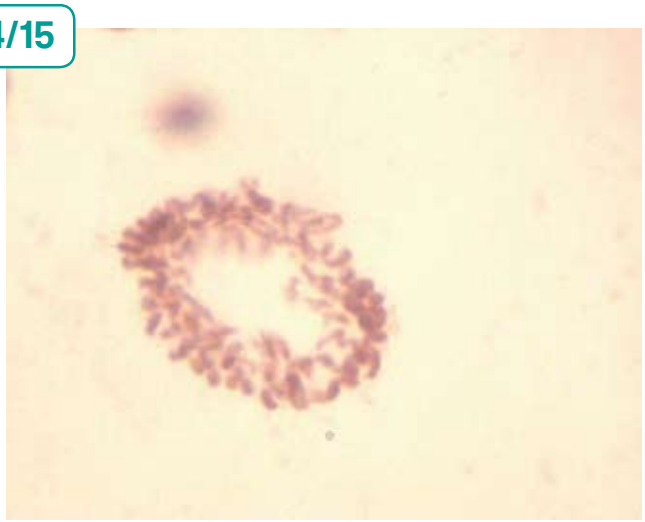

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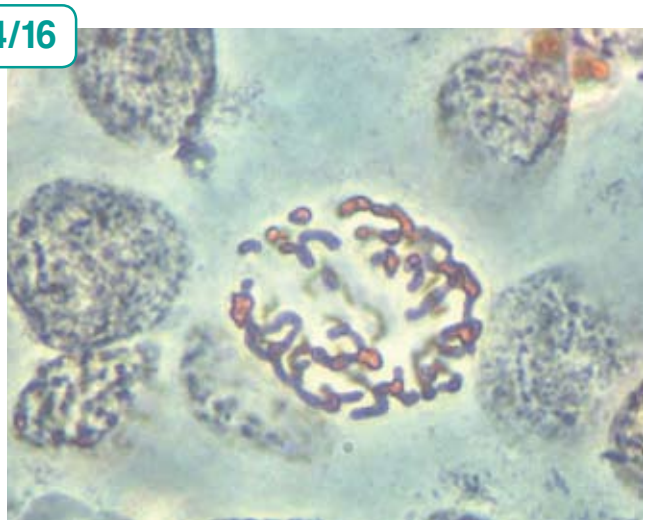

Figure 16: phase contrast

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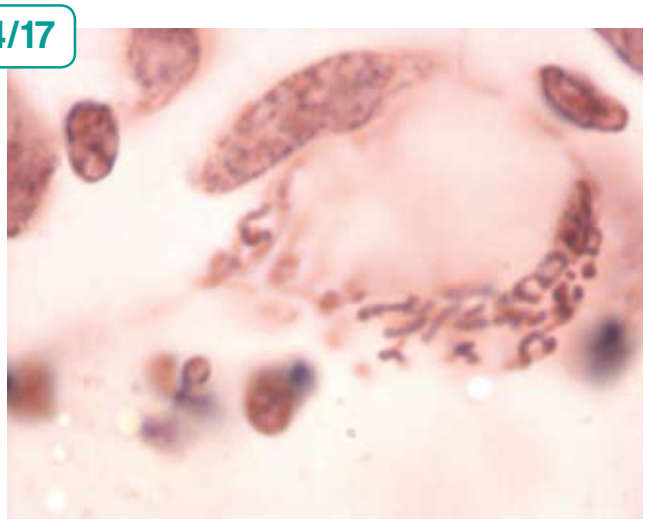
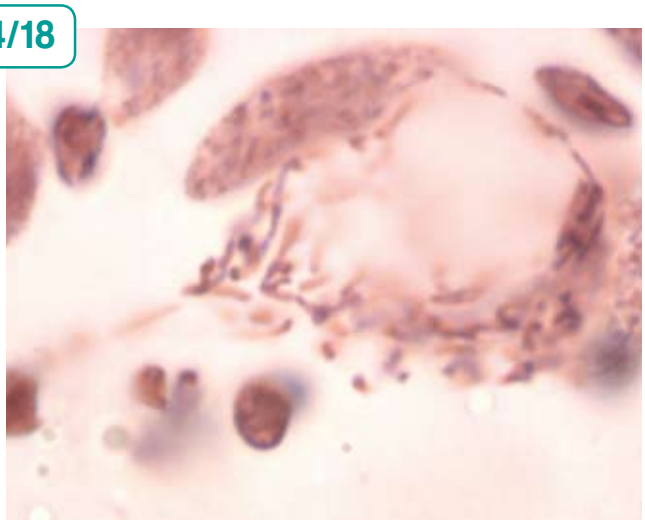
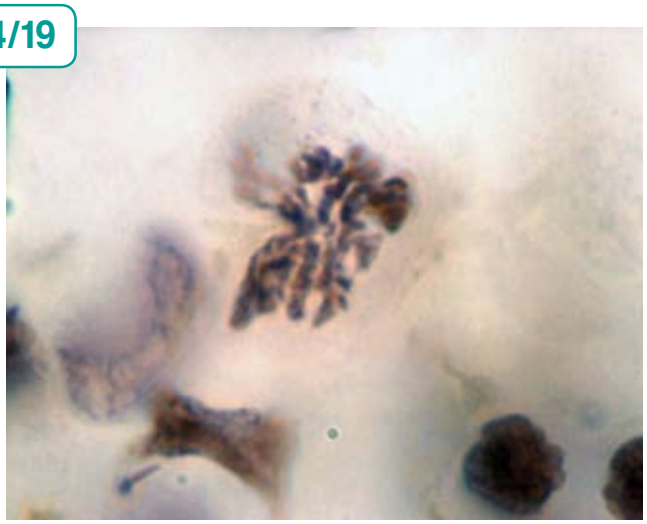


4/18



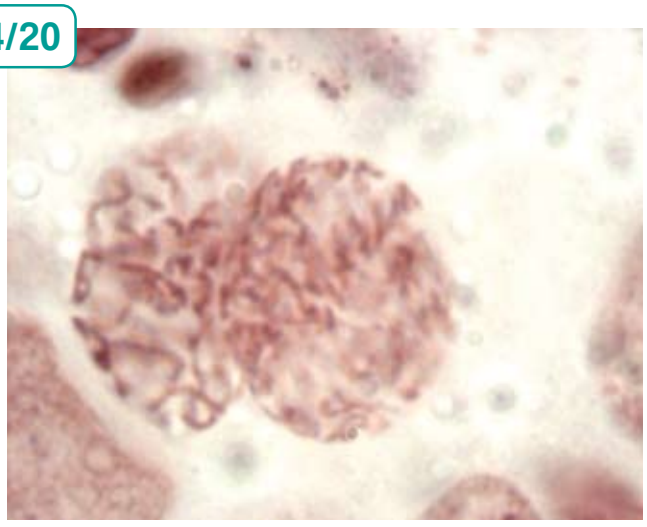
➔ **Metaphase:** chromosomes aligned on the metaphase plate (Figure **4/19**)

4/19

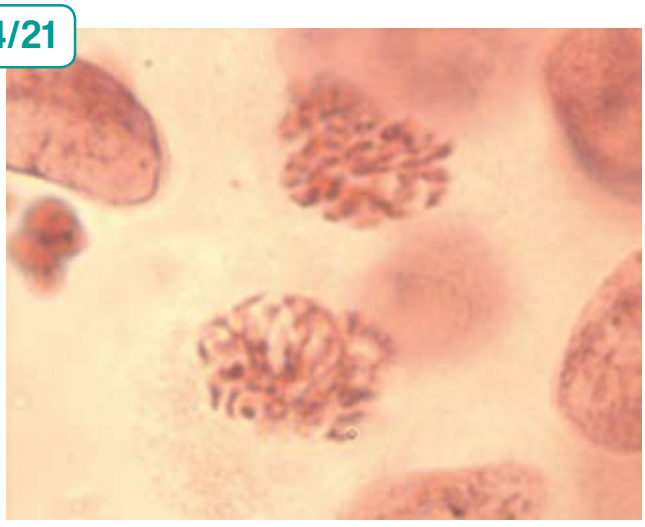


➔ **Anaphase.** Cell division. Each chromosome separates and proceeds toward oposites poles (Figures **4/20** to **4/23**)

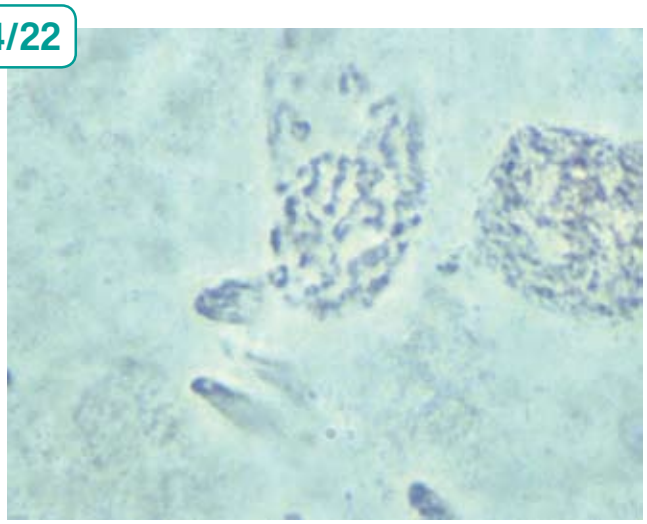
4/20



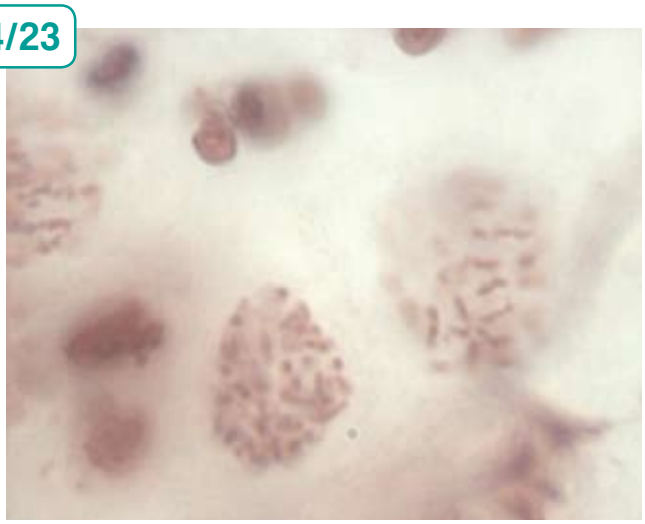
4/21



4/22

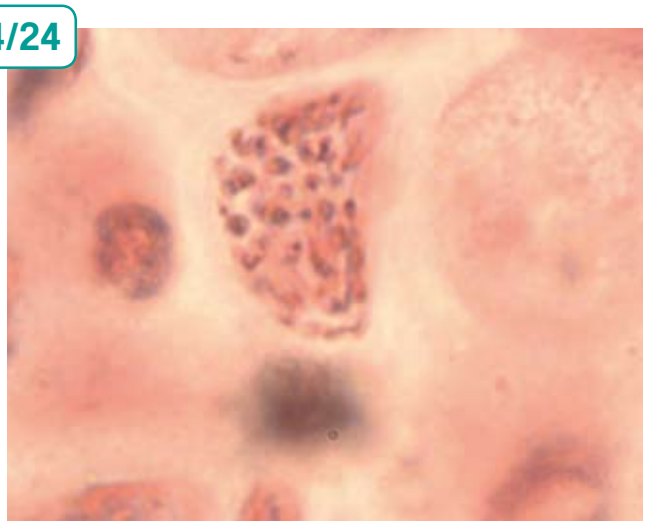


4/23



→ **Telophase:** compact mass of chromosomes beguin to swell and appear to form a small vesicle, that rapidly fuse together to form a convulates tabule (Figures **4/24** to **4/30**)

4/24



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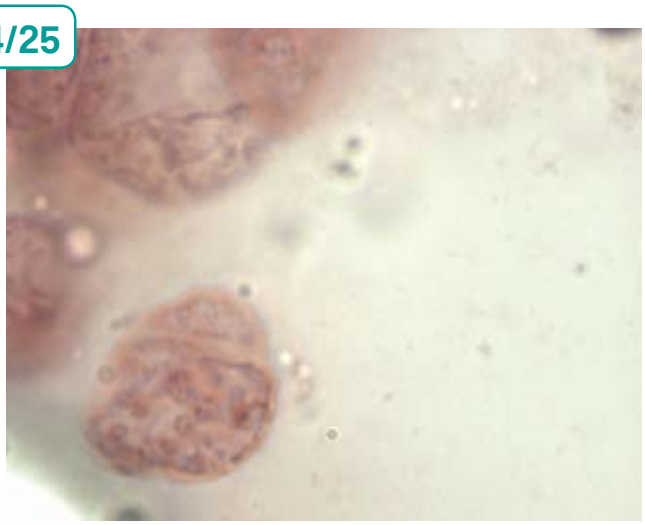

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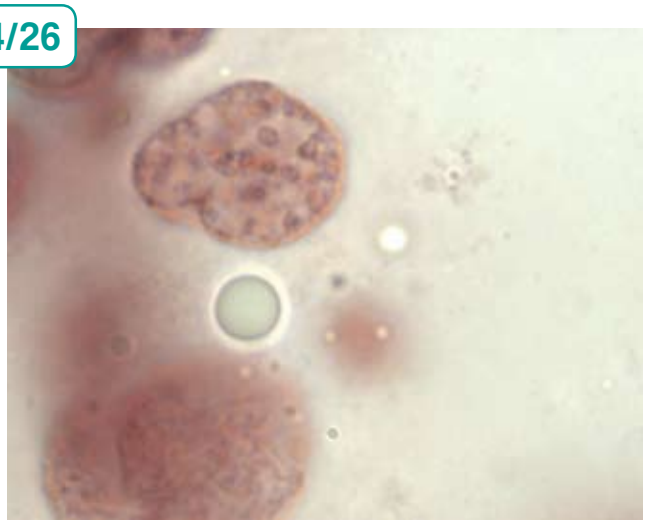
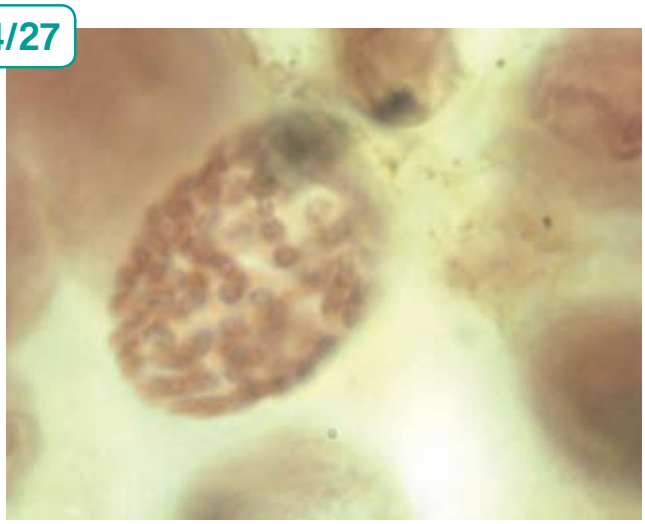


4/27



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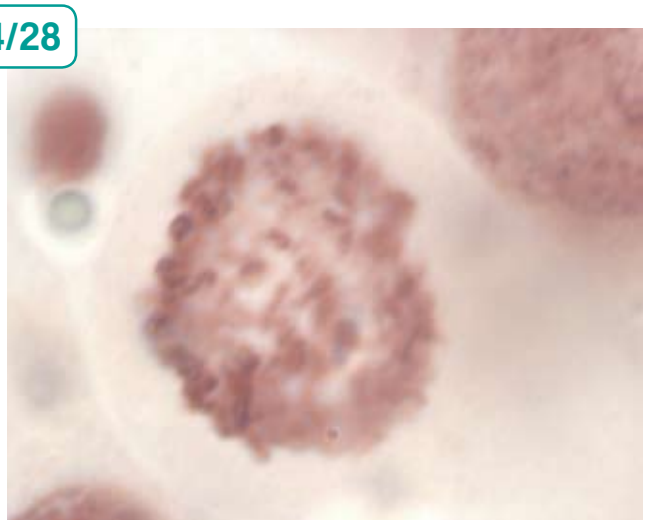

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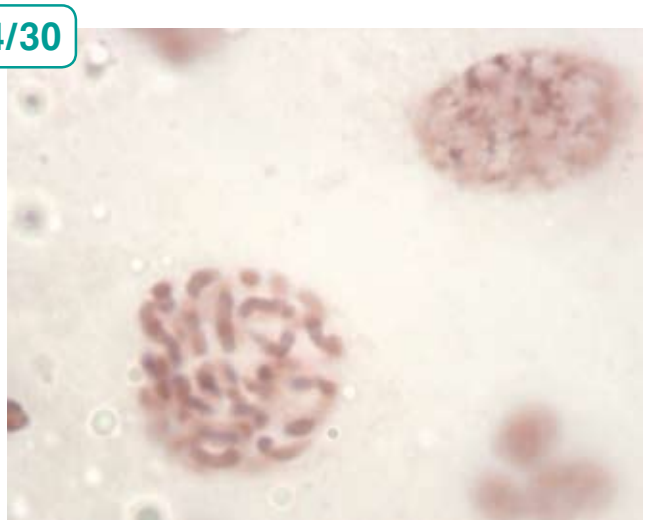
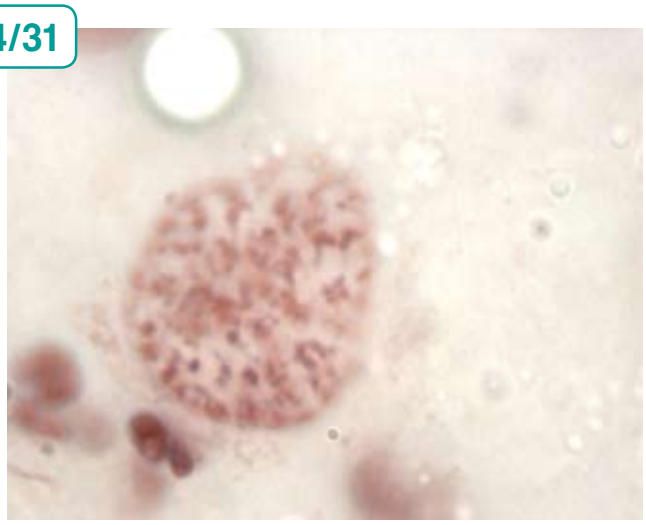
4/30



➔ **Late stage of telophase:** some nucleus still shows tubules structure, but others mimic reticular prophase appearance (Figures **4/31** to **4/33**)

4/31



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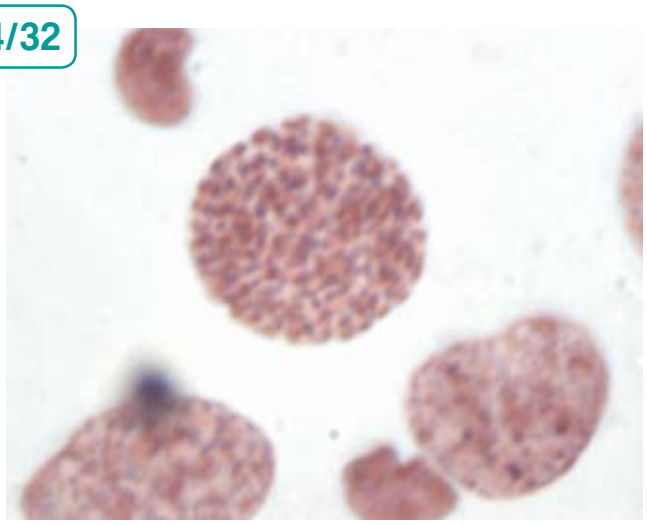
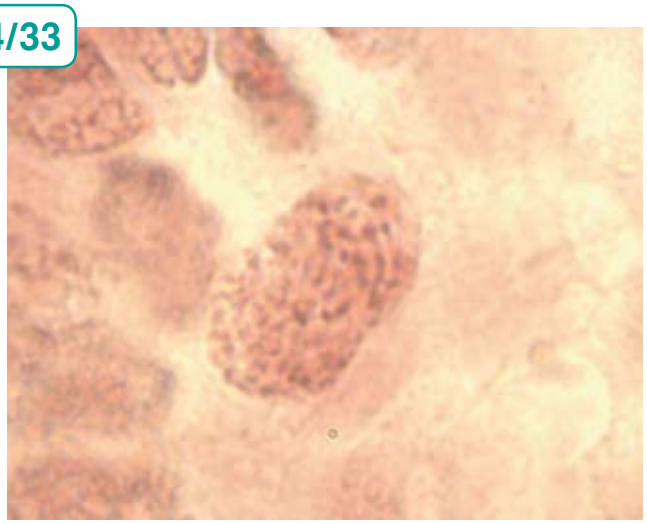
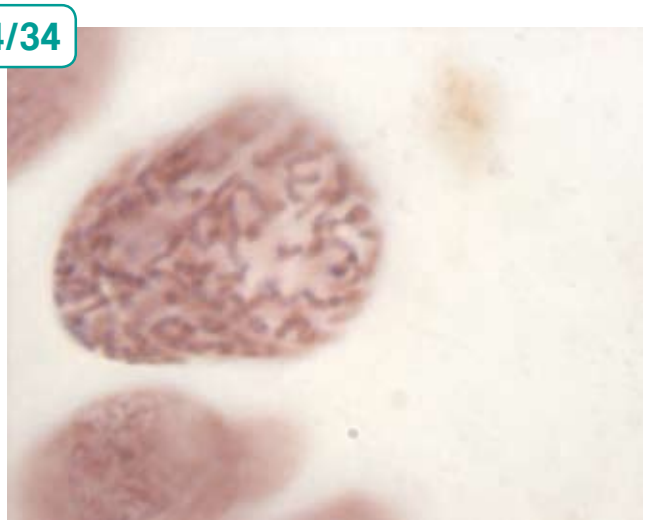


4/33

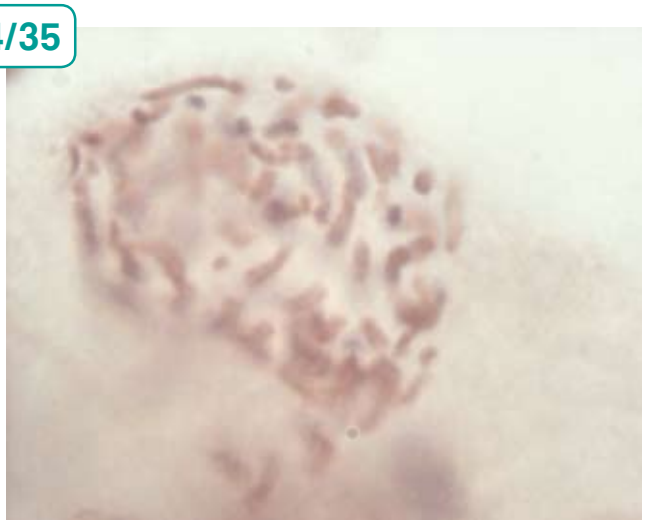


→ **Examples of bipolar cell divisions** (Figures **4/34** to **4/39**)

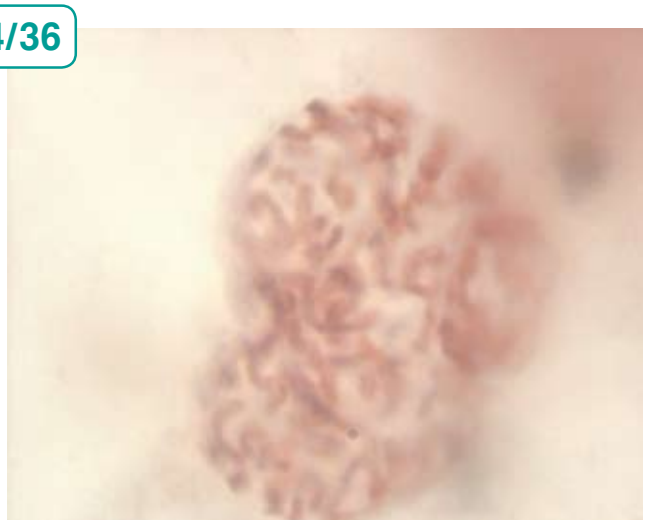
4/34



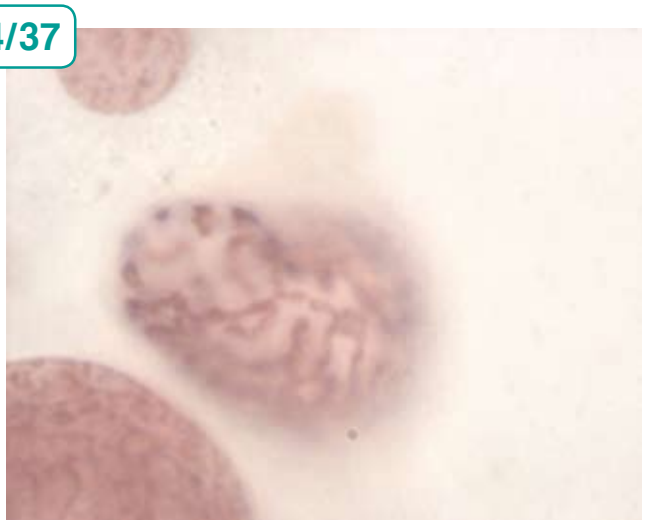
4/35



4/36



4/37



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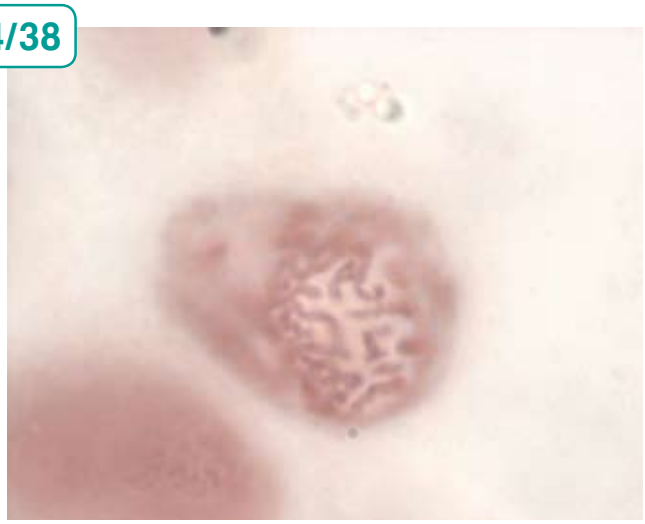

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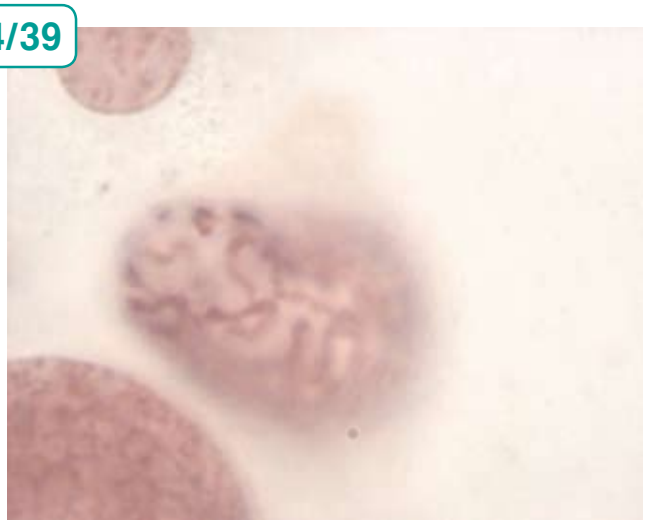
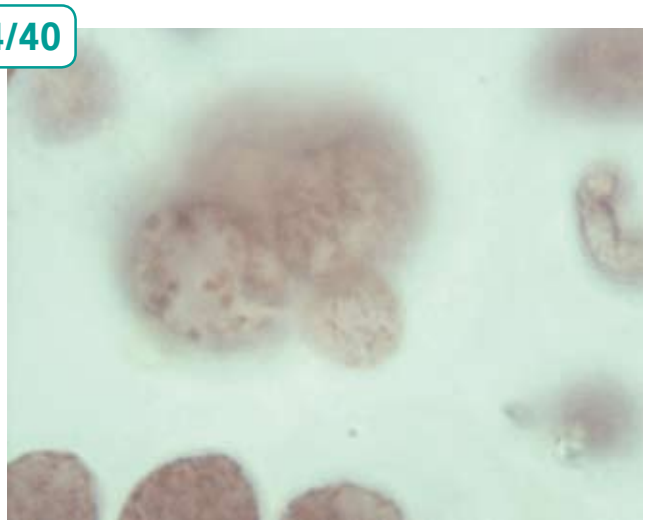


→ **Two examples of multipolar cell division,** with at least four new nuclei per cell (Figures **4/40** to **4/42** and **4/43** to **4/45**)
-Figures 4/37 to 4/39, 4/40 to 4/42 and 4/43 to 4/45: the same nuclei photographed at three focal levels-

4/40



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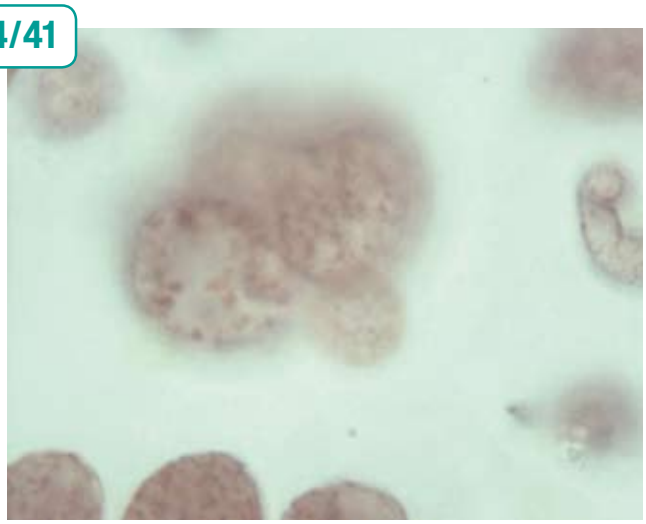
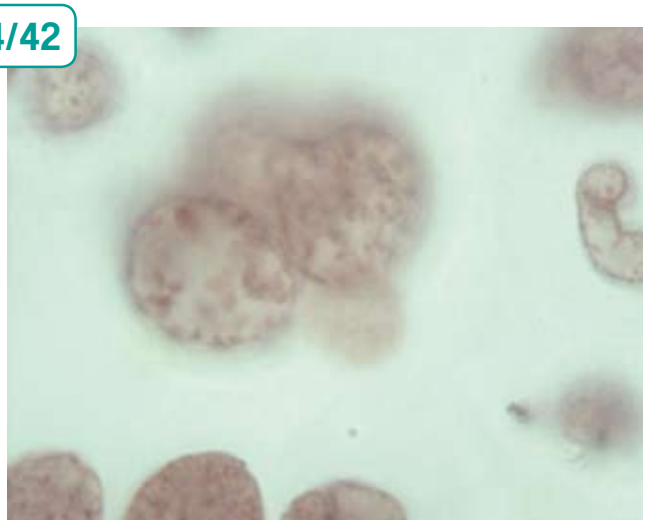


4/42



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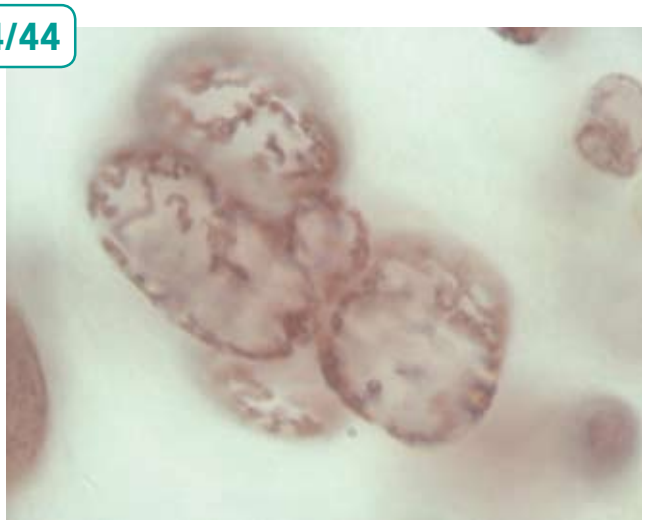
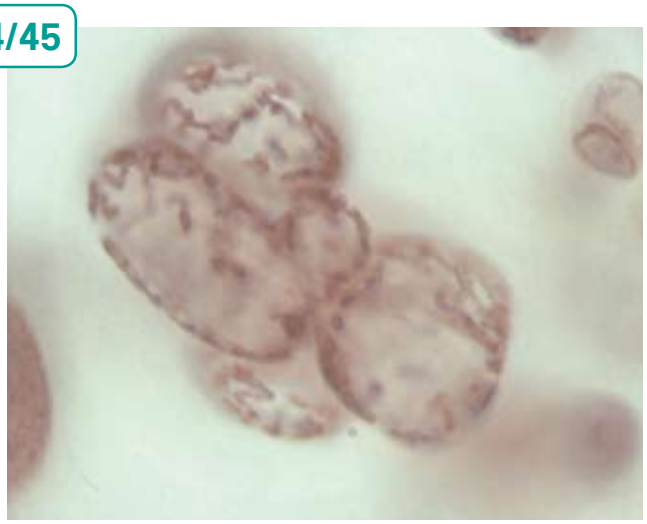
4/44

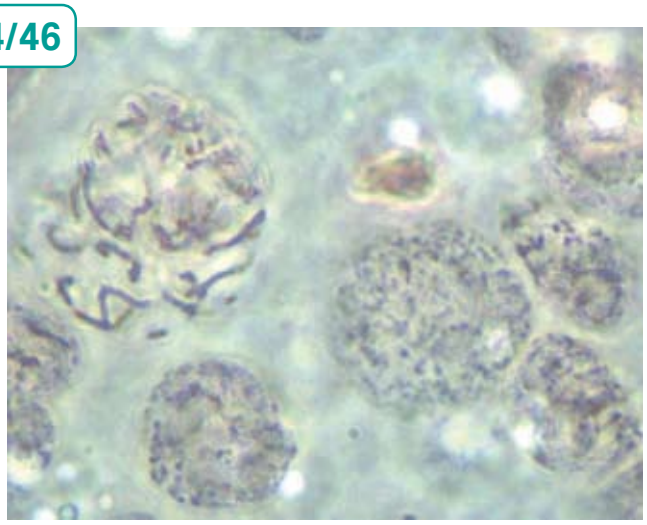


4/45



→ **Lacto-orcein staining (phase contrast).** No cocobacilli can be seen during mitosis, but they can be observed inside neighboring resting cells (Figure **4/46**)

4/46



CHAPTER

5 CRUSHING CELLS: COCCI EXTRACTION

1. Introduction

The true nature of the cocci and rods present inside tumor cells needs to be identified. To assess their presence, improve their observation, and make new tests in order to investigate their characteristics, they need to be extracted to the exterior of the cell. A simple way to do so is using mechanical procedures. Breaking cells by chemical methods injure coccobacilli and, for that reason, it is more useful to interrupt cells mechanically, with a simple smashing.

2. Materials and Methods

Using the sterility protocol described in Chapter 3, samples (from neoplastic and normal lungs) from cases 24 and 25 (Table 2/1) were crushed between two microscope slides (both were protected by two wood plates) with a clamp. All material was sterilized and processed into a laminated-flow cabin (Telstar bio II A/P). After crushing, smears were stained with lacto-orcein and Gram, following procedures described previously.[1] Gram samples were observed with light microscope (×2000) and with phase-contrast in lacto-orcein staining. Cells' content was irregularly distributed after the smashing procedure, it was therefore necessary that microscopic fields were chosen randomly. A transparent panel was placed on the computer screen that provided the microscope images. The panel had 108 squares, with an area per square of 4 cm^2 at ×2000. Using random numbers (generated with Microsoft Excel), nine of 108 squares were selected and the number of extracellular coccobacilli after their extraction from cells was measured.

We studied 30 smears from tumors and another 30 from normal lung tissue, counted coccobacilli per square, and measured their length with Soft Imaging system (Olympus Co Measure).

3. Results

After compression and crushing of cells, cocci and rods could be seen outside and inside the cells. The extracted coccobacilli obtained from tumors were much higher in number than those from normal lung. Lacto-orcein staining showed 3–7 times more extracellular and intracellular coccobacilli in tumors, without statistically significant differences in their lengths (see Table 3/5). Gram staining showed many Gram-positive cocci with clear borders, constant shape, and similar aspects between intracellular and extracellular locations in tumor cells. Rods are actually several cocci very close to each other or cocci during binary division. The mean diameter of cocci, with Gram staining was 0.35 µm, just half of the obtained "bacilli" length with orcein ($p < 0.05$; Table 3/5).

4. Discussion

The neoplastic cells studied here represented the most usual lung tumors: squamous-cell (Figures 5/1 to 5/11) and adenocarcinoma (Figures 5/12 to 5/23). All tumor cells contained coccobacilli on lacto-orcein staining. Both types showed two morphologies of coccobacilli: little round dots, clearly observed but very difficult to measure, and rods with a length of about 0.8 µm. After crushing the cells, such morphologies were also present, outside the cells.

[1] Ardner P, Provine H. *Manual of acute bacterial infections. Early diagnosis and treatment.* 3rd ed. Boston: Little, Brown Co (Inc), 1975: 169.

This finding means that these structures were not artifacts resulting from the addition of several intracellular images. Intact cells stained with orcein had in average about a dozen rods and numerous cocci, but these cocci were so thin that they could not be adequately counted or measured. After their extraction from cells, they were observed better, and it was easier to be quantified when located near broken cells (see Figures 5/1 to 5/3, 5/6, 5/10, 5/12 to 5/21) or when still located inside the cells, because these were less crowded and cells were thinner after mechanical injury (see Figures 5/4, 5/5, 5/7 to 5/9, 5/11, 5/21 to 5/23). By contrast, benign cells extracted only a few dots with irregular sizes and shapes (see Figures 5/24, 5/25).

Gram staining led to the counting of about 100 cocci inside the cells and to nearly 200 around compressed cells (see Figures 5/28 to 5/37). It is important to emphasize that some rods observed with orcein staining were actually cocci when observed with Gram staining, either cases cocci of located very close to each other or cocci during binary division. As matter of fact, the diameters of the cocci measured were approximately half the diameter of such "rod", because this measure results from two cocci.

The experiments[2] with Gram staining showed that: (a) the structures observed have a wall rich in peptoglycans, unlike human cells (see next chapters), because of their affinity to Gram staining; (b) rods observed with orcein staining were cocci, indicating that there is only one morphology inside neoplastic cells; and (c) cocci could be clearly measured because of their constant morphology.

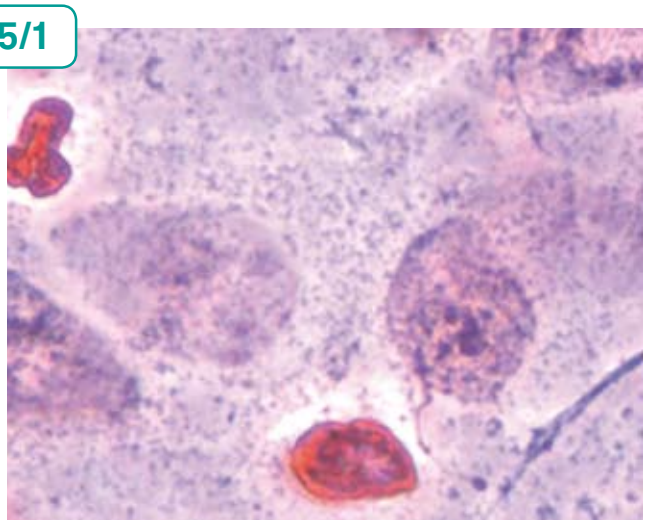
In conclusion, the presence of abundant cocci inside cells is a novel characteristic of cancer cells, not yet recognised in pathology, and it is related statistically with malignancy trait. These numerous cocci, with similarities in shape and size and high affinity to Gram staining, certainly ruled out chromosome pulverisation as a cause of the presence of cocci and suggest the presence of endosimbionts inside tumor cells.

[2] Gladwin M, Trattler B. *Clinical microbiology made ridiculously simple.* 5th ed. Miami: Medmaster, 2011: 1–2.

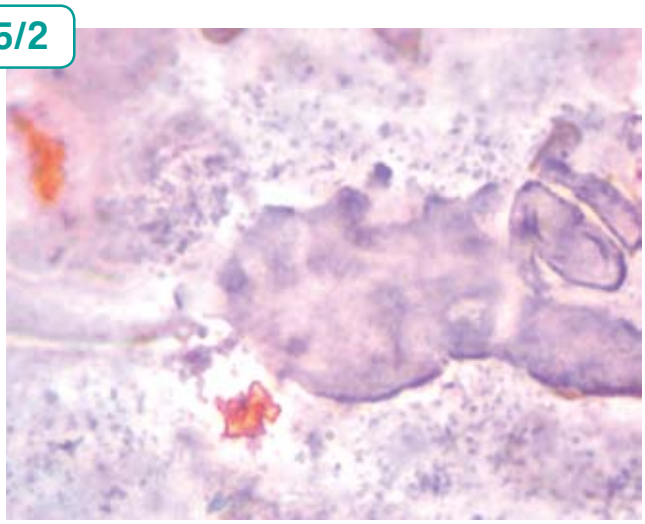
Post mechanical compression.
Osmium-lacto-orcein preparation, dark phase contrast (×2000).

→ **Squamous cell carcinoma** (Figures **5/1** to **5/11**)

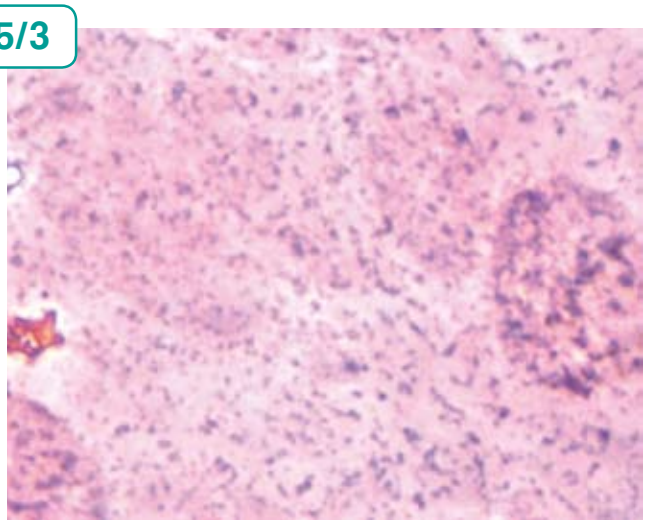
5/1



5/2



5/3



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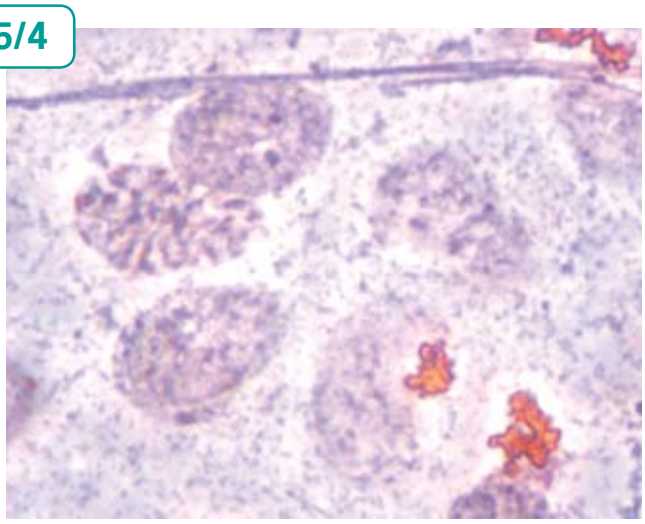

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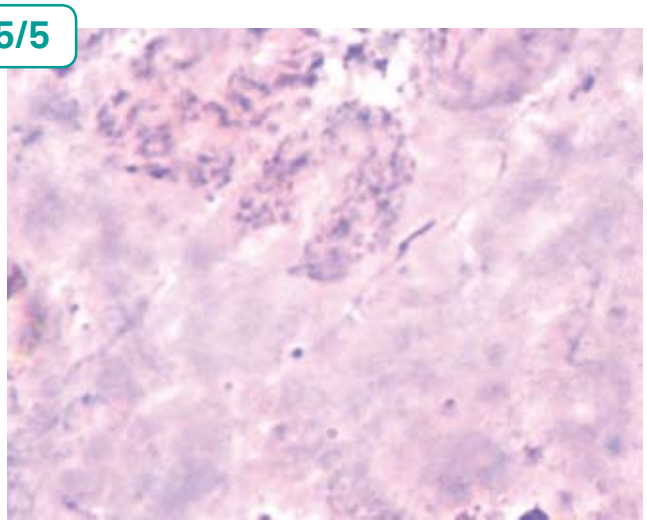

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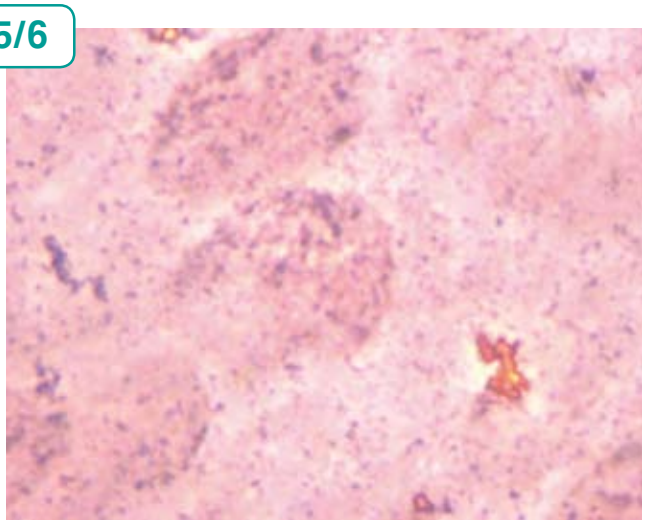

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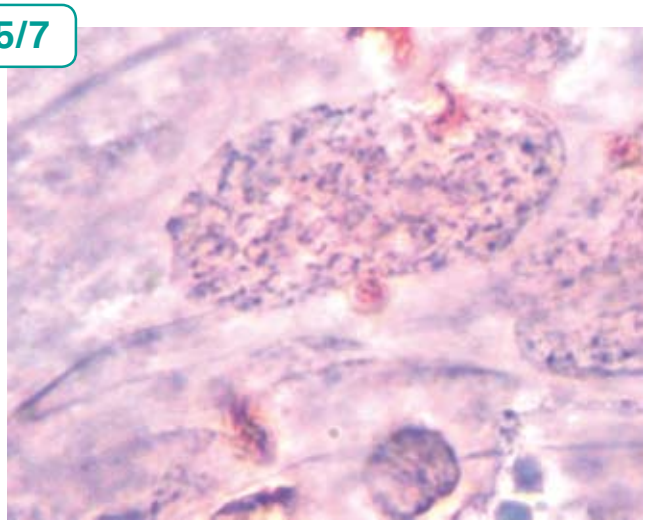

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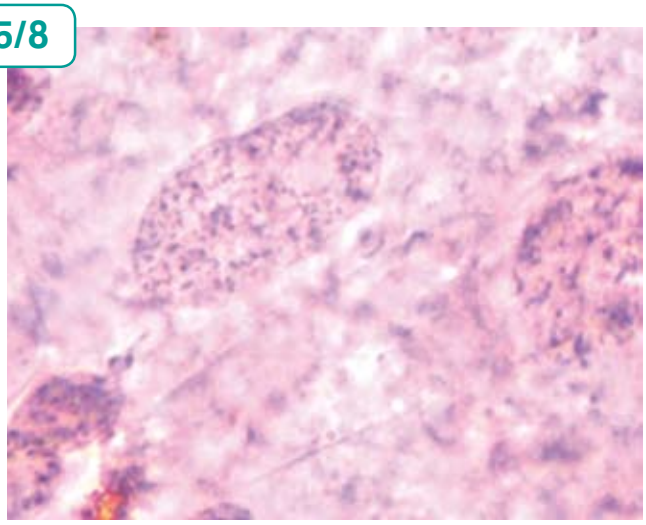

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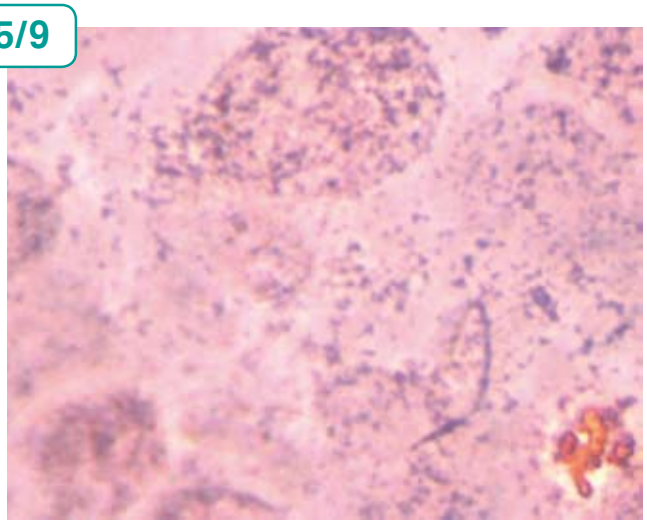

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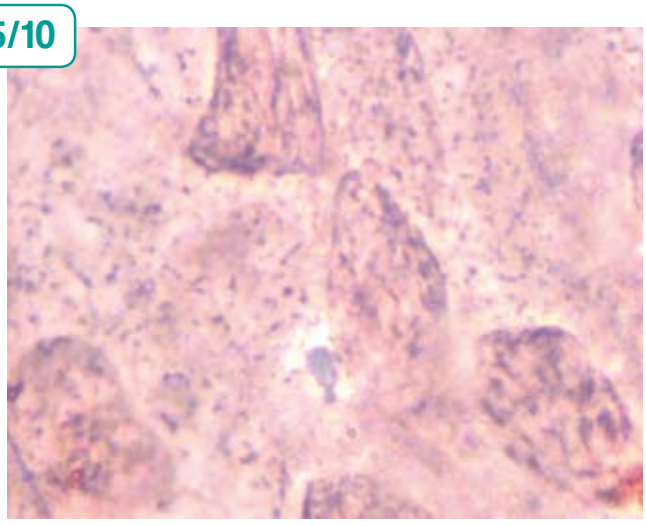

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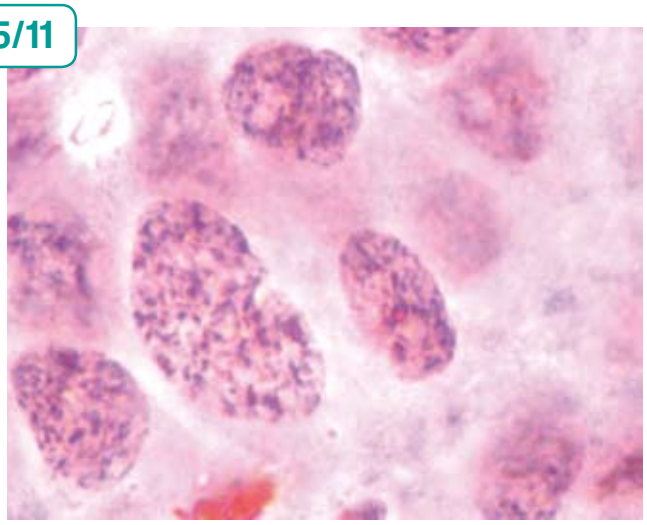
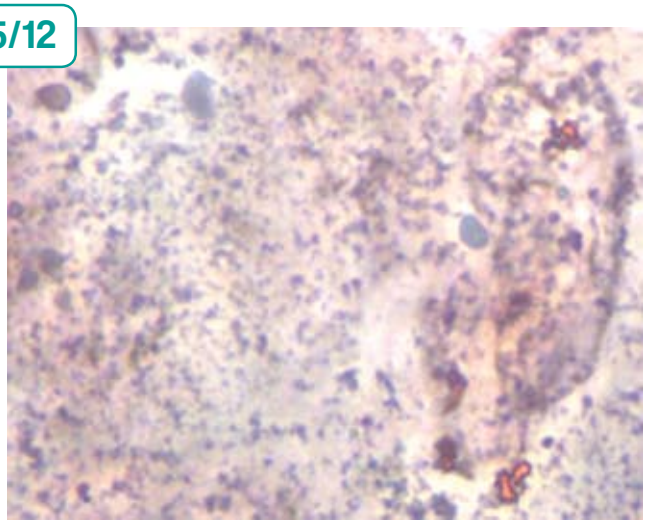


→ **Adenocarcinoma** (Figures **5/12** to **5/23**)

5/12



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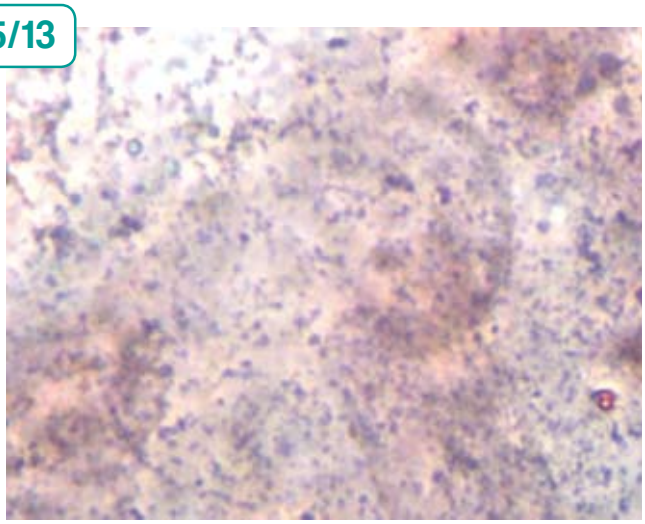

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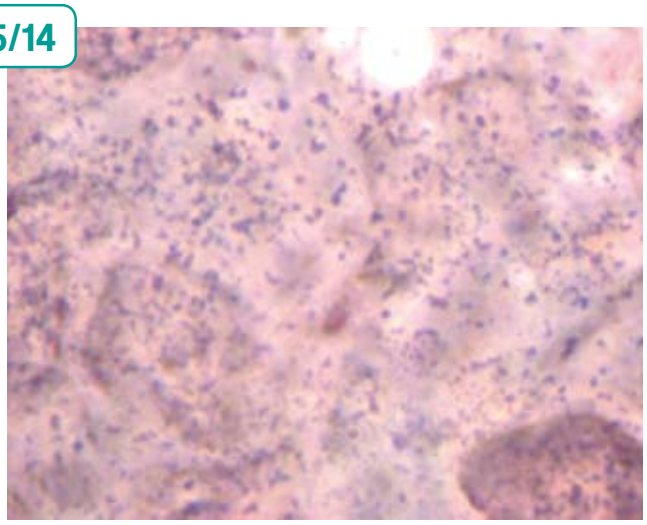

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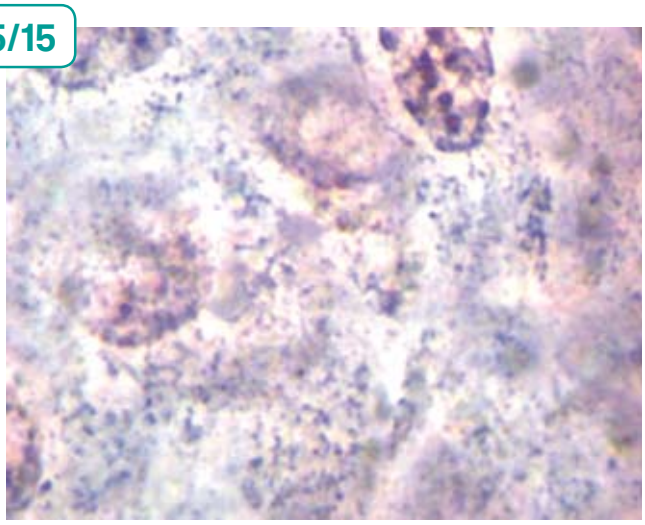

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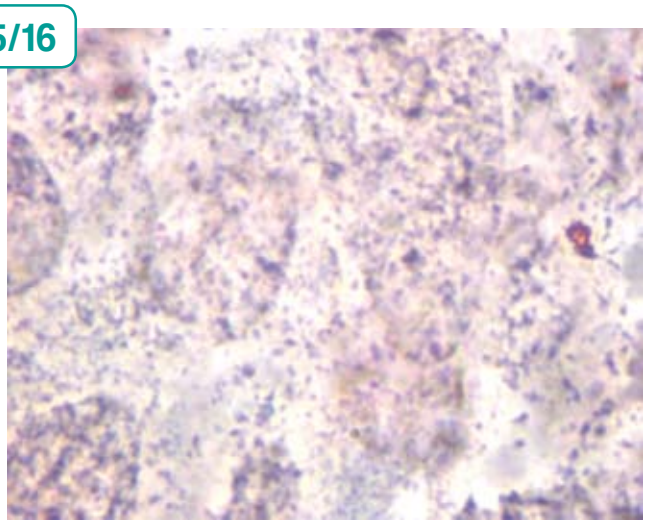

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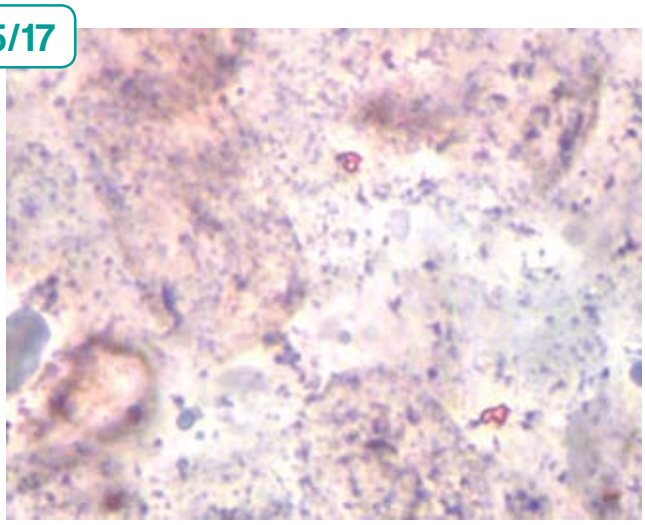

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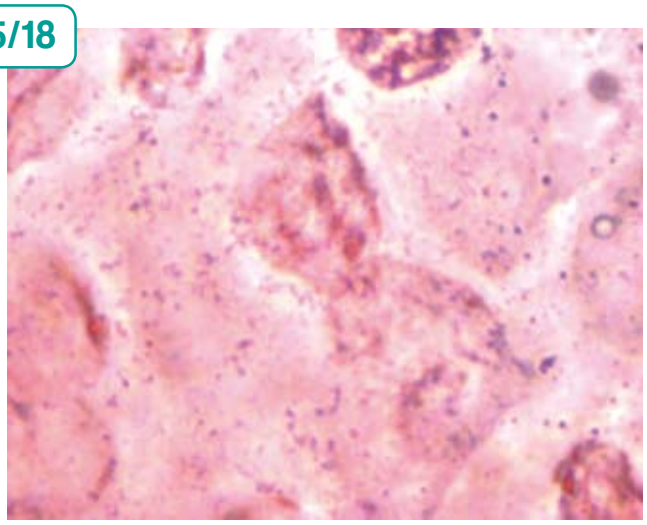

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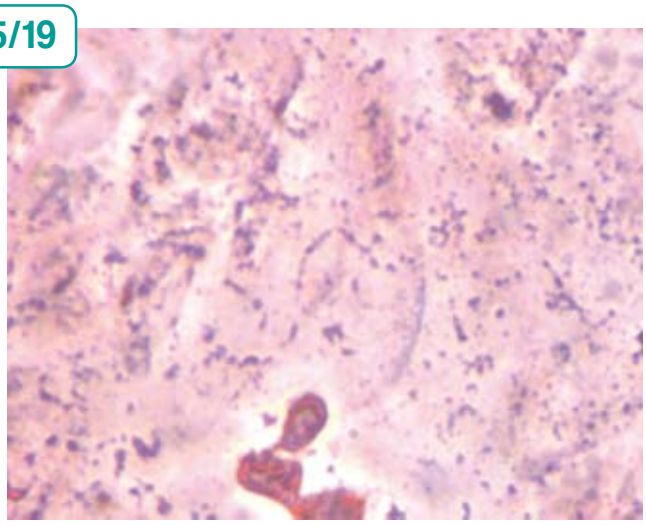

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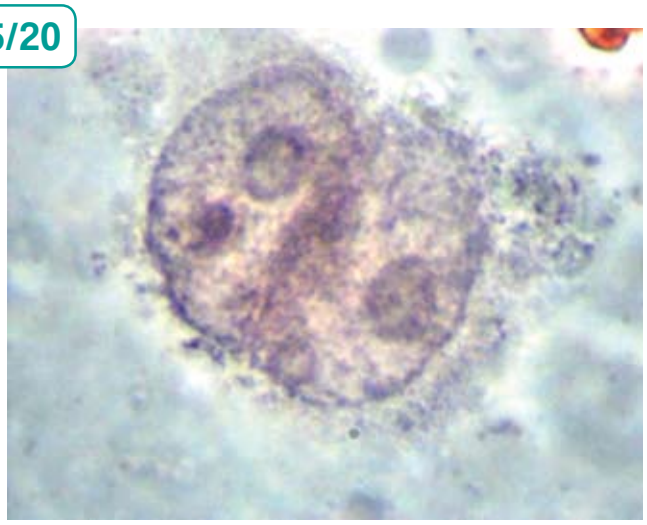

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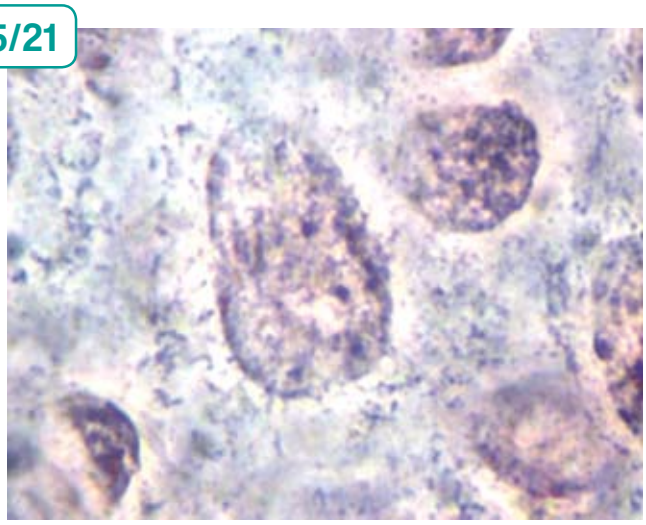

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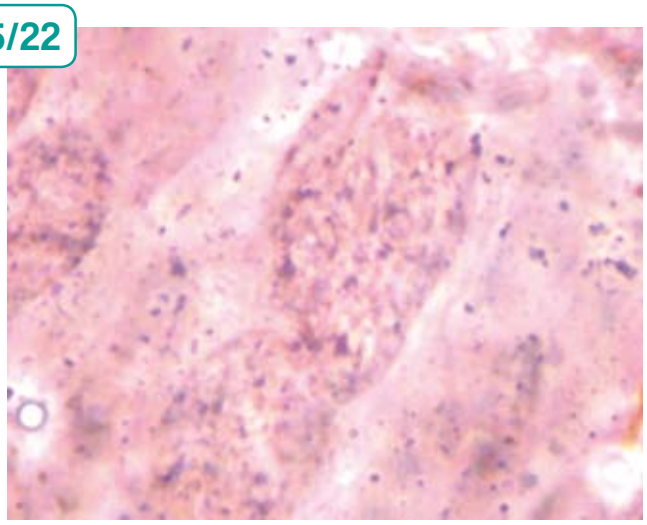

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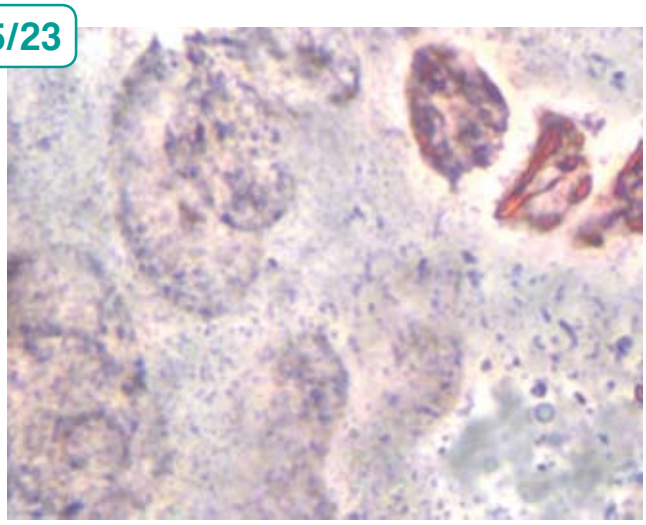
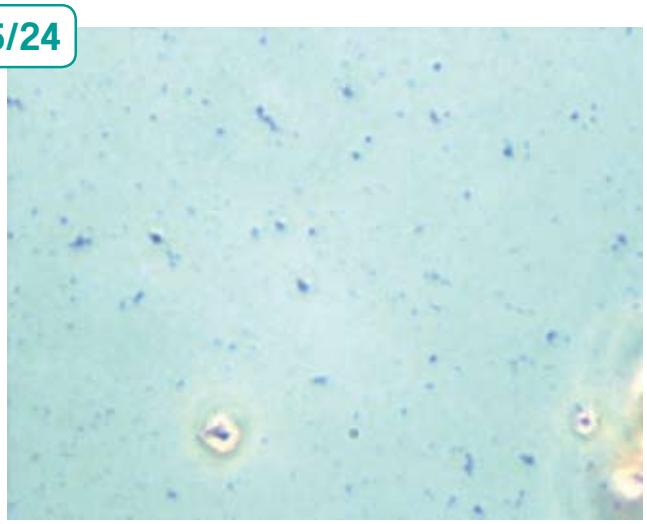


→ **Non tumoral lung samples post-compression** (Figures **5/24** to **5/25**)

5/24



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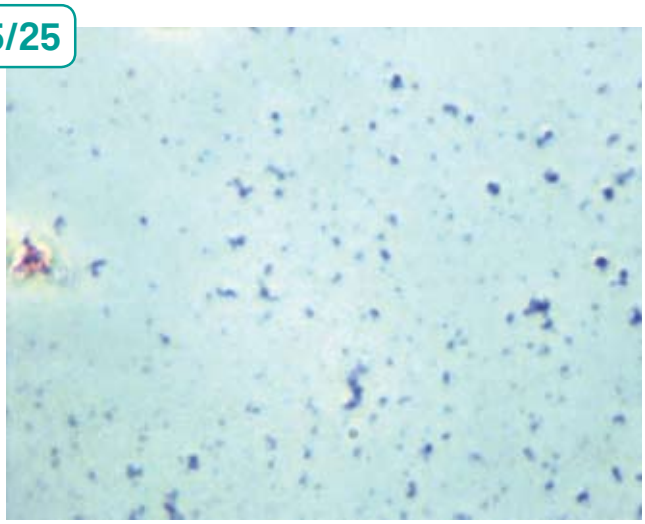
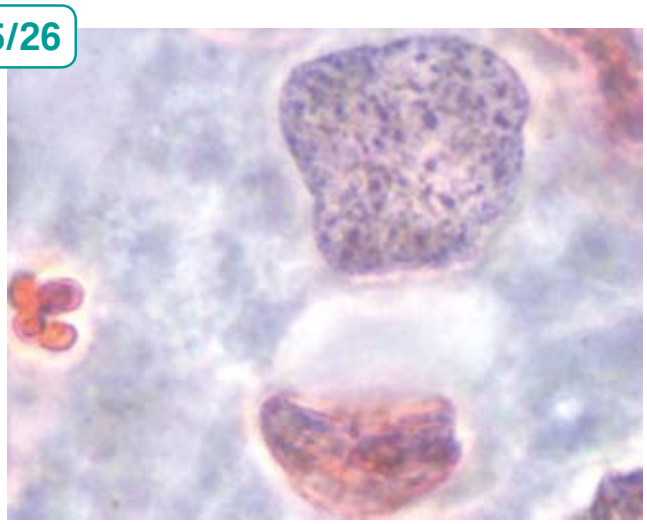


→ Figures **5/26** to **5/27** (lacto-orcein) and **5/29** (Gram) are from the same tumoral sample to comparing extra-cellular cocci

5/26



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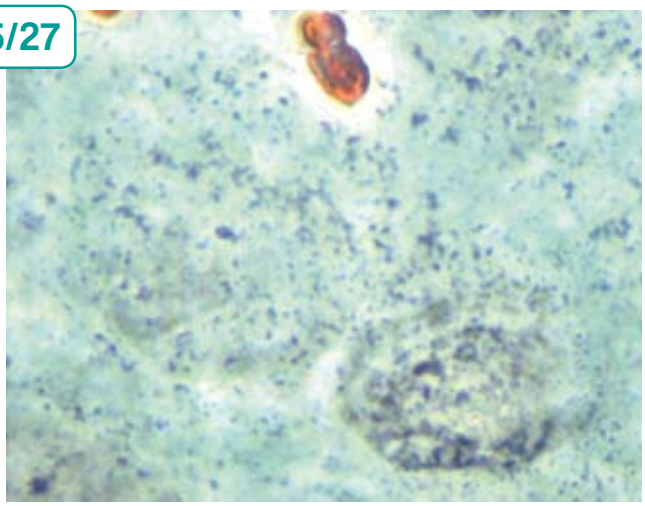

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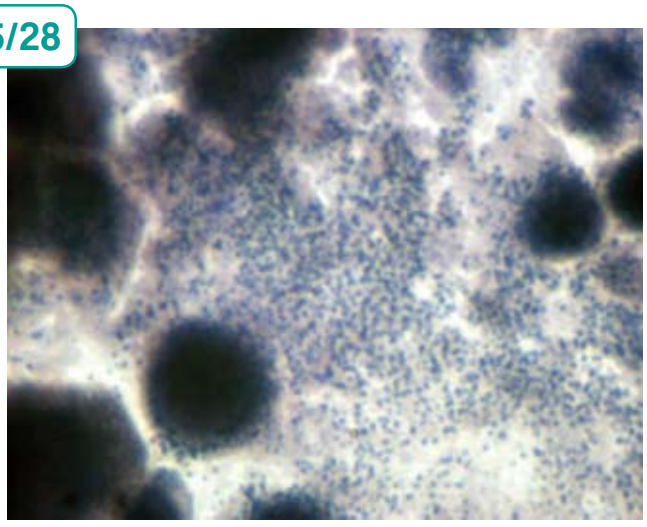

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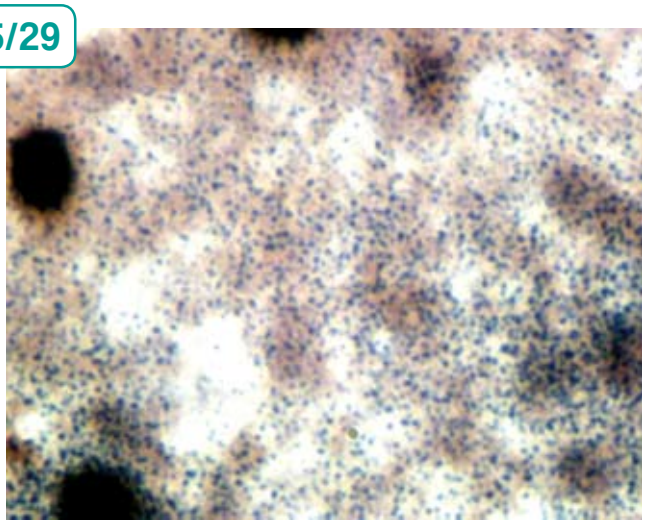
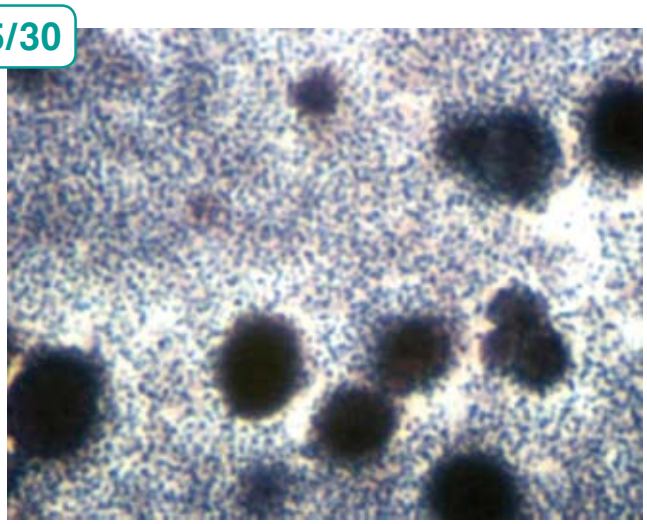


→ **GRAM staining** (Figures **5/28** to **5/37**)**.** Whole preparation containing numerous Gram + cocci. Rods are really very close cocci (Figures **5/36** and **5/37**)

5/30



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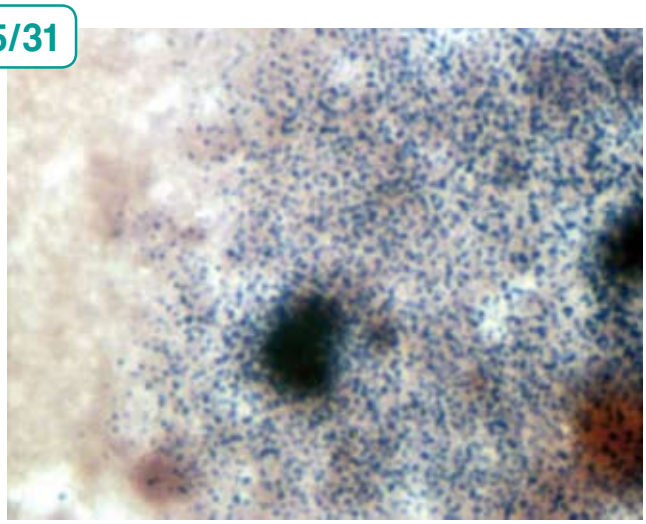

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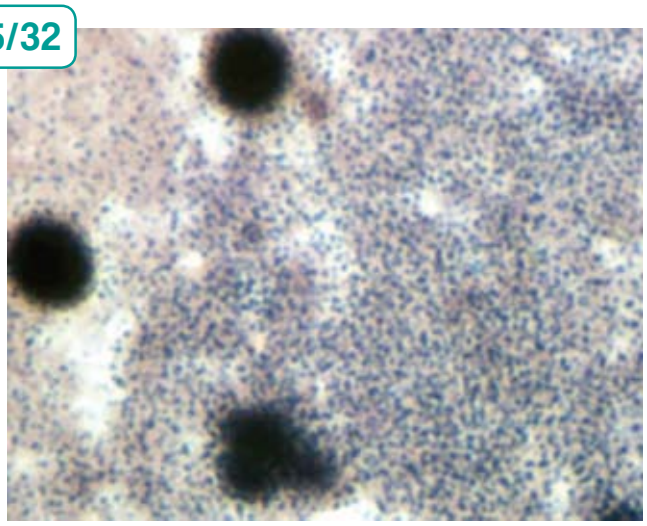
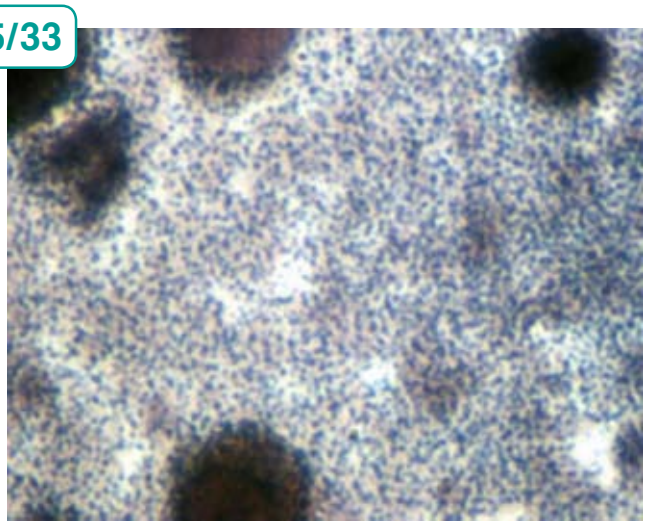


5/33



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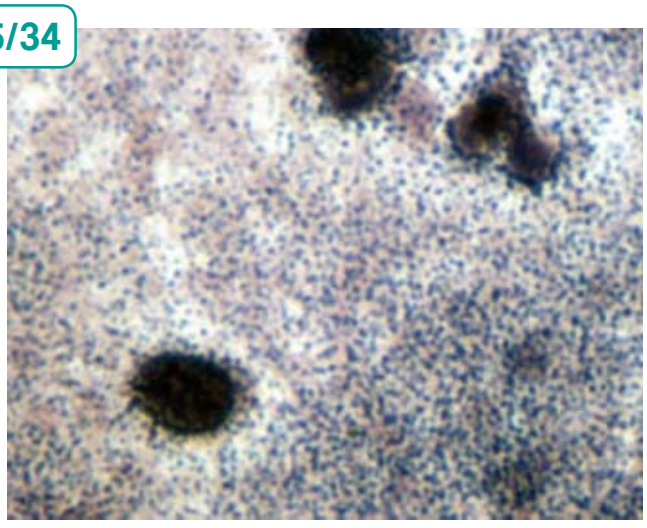

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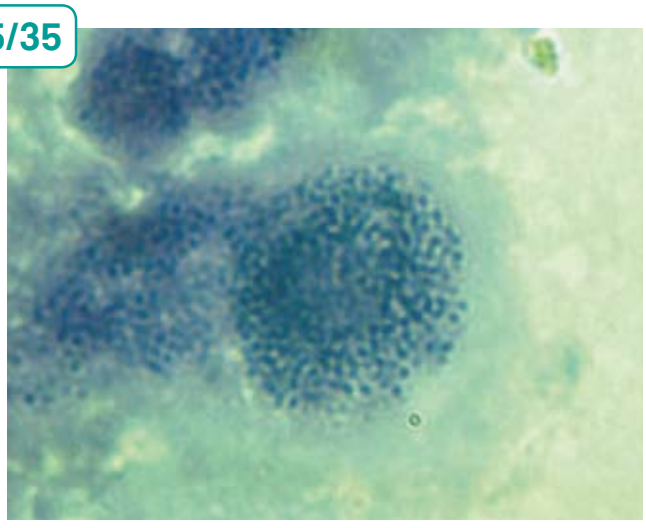
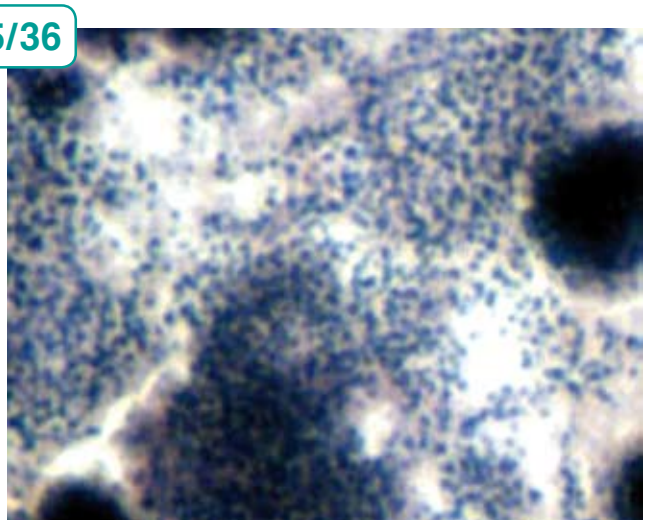
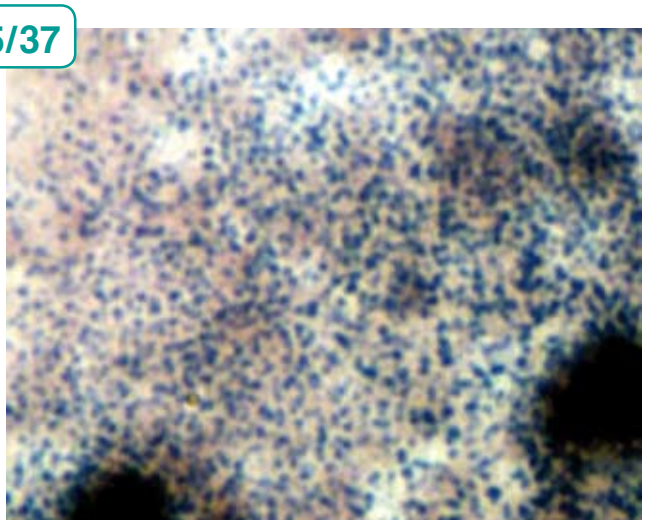


5/36



5/37



CHAPTER 6

GRAM STAINING: INTRACELLULAR GRAM-POSITIVE COCCI

1. Introduction

The presence of assumed bacteria on lacto-orcein stained samples, observed and stained after their extraction, needed to be confirmed inside preserved cells with Gram staining. This observation had to be by comparison of malignant neoplasm with benign cells obtained from the same surgical procedure.

2. Materials and Methods

Using the same aseptic conditions as described in Chapter 3, specimens from malignant neoformations and non-tumor lung cells were processed, immediately after surgery, from cases 25–56 (Table 6/1). They consisted of 31 cancers and 33 non-tumor lung pieces, because one of the patients with a suspected malignant tumor had an inflammatory pseudotumor, diagnosed after surgery procedure (Table 6/2). The smears were Gram stained according to the already described technique.[1] Sterilised slides without cells were also stained with same Gram technique to rule out any artifacts (control samples). Before microscopic observation, it was mandatory to ensure that the coverglass was tightly attached to the slide, because when the stain becomes dry, the coverglass tends to separate from it, making it impossible to correctly observe it on the microscope.

Pictures of both non-tumor and neoplastic Gram-stained cells were randomly allocated and then observers (one expert and one inexpert person who was given a short explanation) tried to identify benign or tumor cells, only by the presence or absence of intracellular Gram-positive cocci, and without any other information.

A cell was considered tumoral when Gram-positive cocci were only seen intracellularly. Cocci or rods outside cells, despite their number, were of no useful in the recognition of cancer cells, because these samples were not mechanically crushed as described in Chapter 5. Smears were studied with a light microscope (×2000) and measurements were obtained following the methods described on Chapter 3. Diameters of 117 extracellular and 42 intracellular cocci from tumors were measured following methods described in Chapter 3. Smears from tumor and benign cells were tested for catalase reaction according to methods described in the literature.[2]

3. Results

Gram staining showed many Gram-positive cocci inside tumor cells, confirming the findings described in Chapter 5. Moreover, it asserted that the observed rods with lacto-orcein staining were actually cocci located very close to each other, or splitting cocci at the cell mitotic division. Thus, from this point onwards, we can say that there is only one morphology inside neoplastic cells, monotonously repeated on different types of squamous cells and adenocarcinomas: Gram-positive cocci. Other less frequent tumors will be studied in Chapter 7.

These cocci completely filled the centre of cells, mainly inside nuclei, and were so close to each other that it was very difficult to calculate their number, which approximated 100 per cell. The shapes of these cocci were quite homogenous, but it is important to note that there were two distinguishable sizes among cocci: those located in nuclei with a diameter of about 0.35 µm (Figures 6/1 to 6/25) and those located intracytoplasmically (Figure 6/27) or extracellularly (Figures 6/26

1 Ardner P, Provine H. *Manual of acute bacterial infections. Early diagnosis and treatment.* 3rd ed. Boston: Little, Brown Co (inc), 1975: 169.

2 Hoeprich Pd. *Infectious diseases.* 1st ed. New York: Harper and Row, 1972: 11.

and 6/28), with a diameter of almost 1 µm. The mean diameter of extracellular cocci was 0.77 µm (1SD 0.22) and that of intracellular cocci was 0.35 µm (1SD 0.10; t student = 5.1, p = 0.000). This size difference will be further discussed in Chapters 8 and 12. Benign cells were stained homogeneously with Gram staining and, generally, no clear cocci or rods were seen in their structures (Figures 6/29 and 6/30).

Gram staining showed intranuclear cocci in 28 of 31 tumor samples and only in three of 33 non-neoplastic samples: two in benign cases and one in the case of inflammatory pseudotumor (Figures 6/31 to 6/36); OR = 93, $\chi^2 = 39$, p = 0.000. The catalase test was positive on tumor, but also on benign cell samples. Controls were free from cocci and rods.

The recognition of benign versus malignant cells, by observation of only intracellular Gram-positive cocci reached nearly 90% sensitivity and specificity when done by the expert (Table 6/3). Very similar results were obtained with the inexpert observer, and both results could not be explained by hazard (p = 0.000).

4. Discussion

Neoplastic cells studied here (squamous-cell and adenocarcinoma) were crowded with Gram-positive cocci and could be observed in nine of ten cells. Results from less frequent tumor types will be studied in Chapter 7. In the two types studied here, there was invariably only one morphology (cocci): cocci of small size located inside the nuclei, and larger cocci located in cytoplasm or extracellular places. Moreover, the presence of cocci-bearer nuclei seems to be strongly associated with cancer risk.

Even after ethanol wash, cocci with smooth and clear edges retained the violet-iodine complex, which reinforces the conclusions of Chapter 4: cocci have cell walls a peptidoglycan layer located just outside the bacterial cytoplasmic membrane (unlike animal cells, which have only a single cytoplasmic membrane composed of a phospholipid bilayer). Moreover, in those tumor cells there are bacterial structures that do not come from human cells and which are related probably to streptococci (see Chapters 10 and 12). The fact that cocci are present in all parts of the cells, but predominantly inside the nuclei can explain, partly, the observed irregular morphology and hypertrophy of cells and nuclei, as well as the increment of their mitosis, described in Chapter 4.

The results so far, from Chapters 3 to 6, lead us to think that cocci-bearer cells seem to acquire new morphometric traits and to have quicker mitosis rates than do remaining normal cells allowing their expansion to the point of replacing most of human tissues and organs. Cocci-bearing nuclei are a unique pathological finding and could be considered a tumoral pathognomonic trait. The spread of tumor is caused by the mobility of cancer cells through anatomical routes (bronchi, blood or lymphatic vessels, etc), but cocci (probably large forms) can easily reach long distance targets thanks to their small size and massive number. A microscopic tumor holds up to 1 million cells, but carries nearly 100 millions of cocci, with the potential capacity to invade many normal cells and to follow the progressive and fatal evolution that occurs in most malignant cancerous cells.

Table 6/1. CHARACTERISTICS OF 32 CASES. GRAM STAINING.

Nº	AGE (years)	GENDER	TYPE OF TUMOR	STAGE			PRIMARY TUMOR		YEAR OF SURGERY	TYPE OF LUNG RESECTION
				T	N	M	Φ MAXIMAL (cm)	AREA (cm^2)		
25	73	FEMALE	ADENOCARCINOMA	2	0	X	3	10.5	2009	NEUMONECTOMY
26	64	MALE	ADENOCARCINOMA	2	1	X	12	144	2009	NEUMONECTOMY
27	56	FEMALE	SQUAMOUS CELL	3	1	X	3.5	17.5	2009	LOBECTOMY
28	54	MALE	ADENOCARCINOMA	1	0	X	2.3	5.75	2009	LOBECTOMY
29	49	MALE	ADENOCARCINOMA	2	1	X	3.9	15.6	2009	LOBECTOMY
30	57	MALE	SQUAMOUS CELL	3	0	X	3	16.5	2009	LOBECTOMY
31	78	FEMALE	SQUAMOUS CELL	2	0	X	2.8	8.4	2009	LOBECTOMY
32	72	MALE	SQUAMOUS CELL	2	1	X	5.5	33	2009	LOBECTOMY
33	79	MALE	SQUAMOUS CELL	2	0	0	3.5	14	2009	LOBECTOMY
34	53	MALE	SQUAMOUS CELL	2	0	X	3	12	2009	LOBECTOMY
35	66	MALE	LARGE-CELL NEURENDOCRINE CANCER	2	1	X	1.8	3.6	2009	LOBECTOMY
36	73	MALE	SQUAMOUS CELL	3	0	X	5	35	2009	LOBECTOMY
37	74	MALE	SQUAMOUS CELL	2	0	X	2.5	8.75	2009	NEUMONECTOMY
38	58	MALE	SQUAMOUS CELL	3	0	X	3.5	15.8	2009	ATYPICAL RESECTION
39	49	FEMALE	LARGE-CELL NEURENDOCRINE CANCER	3	0	0	3.5	14	2009	LOBECTOMY
40	71	FEMALE	ADENOCARCINOMA	1	1	0	2	5	2009	NEUMONECTOMY
41	67	MALE	ADENOCARCINOMA	2	2	0	5	30	2009	NEUMONECTOMY
42	49	MALE	SQUAMOUS CELL	2	1	X	4	20	2010	NEUMONECTOMY
43	55	MALE	ADENOCARCINOMA	3	2	X	2.5	11.3	2010	LOBECTOMY
44	58	MALE	INFLAMMATORY PSEUDOTUMOR	4	X	X	1.5	4.5	2010	LOBECTOMY
45	74	MALE	PLEOMORPHIC SARCOMA	2	0	0	3.5	14	2010	LOBECTOMY
46	59	MALE	SQUAMOUS CELL	2	0	X	1.3	1.95	2010	LOBECTOMY
47	74	MALE	SQUAMOUS CELL	2	0	X	4.5	27	2010	LOBECTOMY
48	58	FEMALE	METASTATIC RENAL ADENOCARCINOMA	2	2	1	4	20	2010	LOBECTOMY
49	70	MALE	ADENOCARCINOMA	2	0	X	3	9	2010	NEUMONECTOMY
50	63	MALE	SQUAMOUS CELL	2	0	X	3	9	2010	LOBECTOMY
51	55	MALE	ADENOCARCINOMA	1	0	X	3.5	15.8	2010	LOBECTOMY
52	76	MALE	ADENOCARCINOMA	2	0	X	4	16	2010	LOBECTOMY
53	78	MALE	ADENOCARCINOMA	2	0	X	3.5	12.3	2010	ATYPICAL RESECTION
54	81	MALE	SQUAMOUS CELL	2	0	X	4.5	24.3	2010	ATYPICAL RESECTION
55	58	MALE	ADENOCARCINOMA	1	0	X	2.2	5.5	2010	LOBECTOMY
56	71	MALE	SQUAMOUS CELL	2	0	X	2	4	2010	NEUMONECTOMY

Table 6/2. SOCIODEMOGRAPHICS AND TUMOR CHARACTERISTICS OF 32 CASES. GRAM STAINING.

AGE (years)	MEAN ± 1SD. (MAX-MIN)	64.75±9.9 (48-81)		
GENDER	FEMALE	6 (18.8%)		
	MALE	26 (81.3%)		
DATE OF SURGERY	2009	17 (53.1%)		
	2010	15 (46.9%)		
TYPE OF TUMOR	ADENOCARCINOMA	12 (37.5%)		
	SQUAMOUS-CELL CANCER	15 (46.9%)		
	METASTATIC RENAL ADENOCARCINOMA	1 (3.1%)		
	LARGE-CELL NEURENDOCRINE CANCER	2 (6.3%)		
	PLEOMORPHIC SARCOMA	1 (3.1%)		
	INFLAMMATORY PSEUDOTUMOR	1 (3.1%)		
TNM DESCRIPTOR		T	N	M
	0		21 (65.6%)	5 (15.6%)
	1	4 (12.5%)	7 (21.9%)	1 (3.1%)
	2	21 (65.6%)	3 (9.4%)	
	3	6 (18.8%)		
	4	1 (3.1%)		
	X		1 (3.1%)	26 (81.3%)
TYPE OF LUNG RESECTION	ATYPICAL RESECTION	3 (9.4%)		
	LOBECTOMY	21 (65.6%)		
	NEUMONECTOMY	8 (25.0%)		

Table 6/3. INTRACELLULAR COCCI. GRAM STAINING (×2000).

READERS	GRAM STAINING	Nº CASES		
		CANCER	NON TUMORAL LUNG	TOTAL
EXPERT	COCCI +	18	0	**18**
	COCCI -	0	27	**27**
	TOTAL	**18**	**27**	**45**
INEXPERT	COCCI +	16	3	**19**
	COCCI -	2	26	**28**
	TOTAL	**18**	**29**	**47**

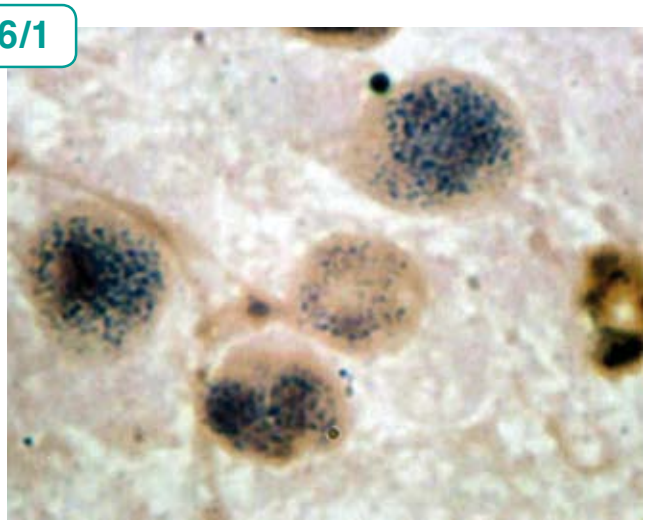
	COMPARISON: EXPERT vs INEXPERT	
	EXPERT	INEXPERT
SENSITIVITY (%) 95% CI	100 90 to 100	88.8 80 to 98
SPECIFICITY (%) 95% CI	100 90 to 100	89.6 80 to 98
χ^2 P VALUE	40.0 0.000	25.3 0.000

Gram-stained smears are seen at ×2000 (oil-inmersion lens)

FIGURES 6/1 TO 6/36

→ **Morphology and characteristics of organism encountered in tumor specimens.** Note that Gram + cocci are present inside cells, preferently into its centres. Rods are really cocci very close or during división process (Figures **6/1** to **6/25**)

6/1



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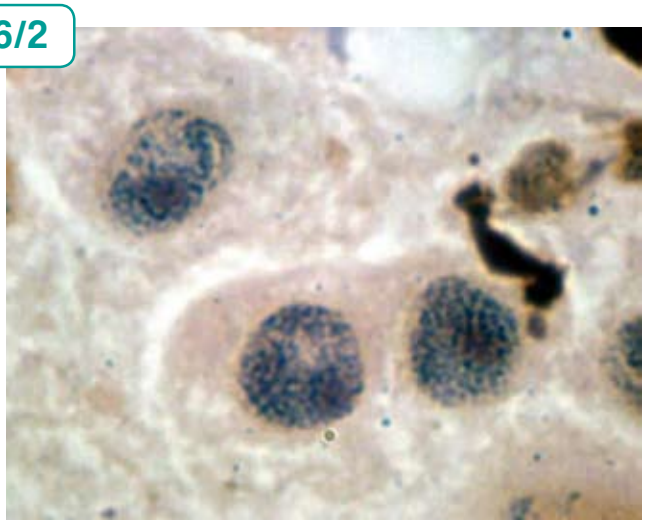

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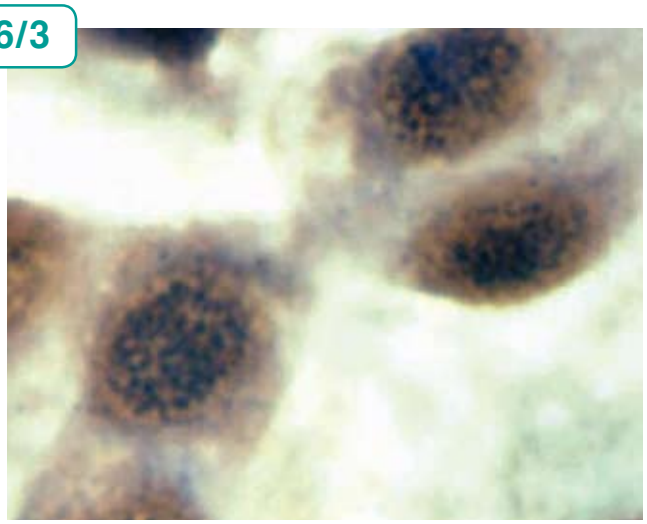

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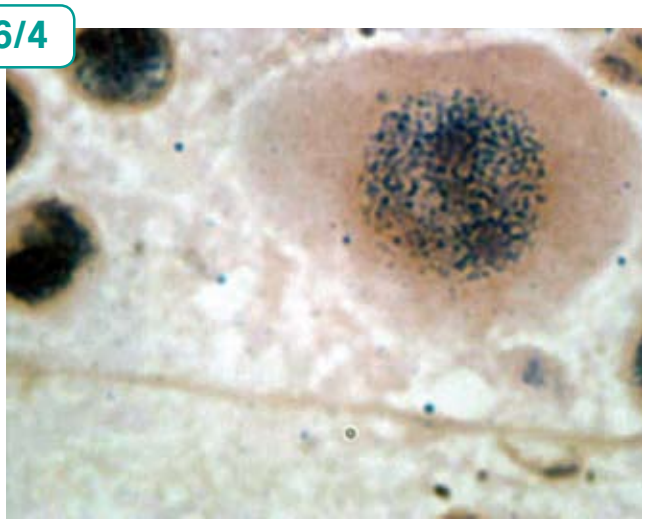

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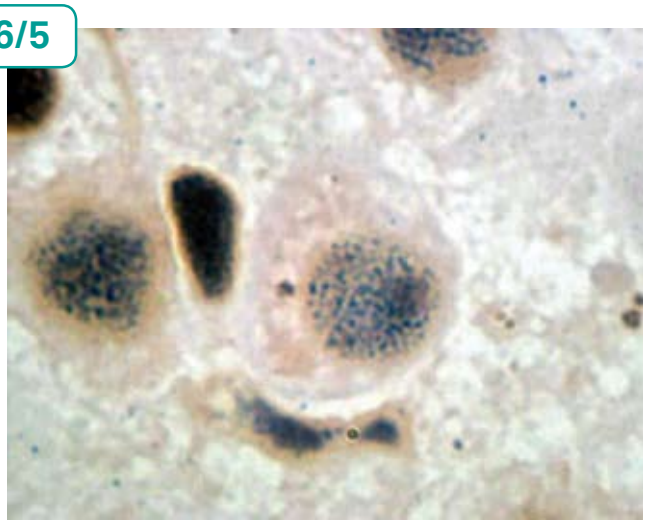

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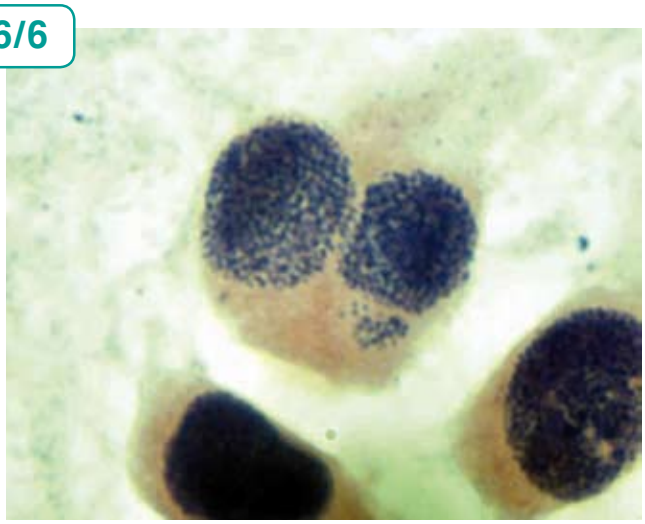

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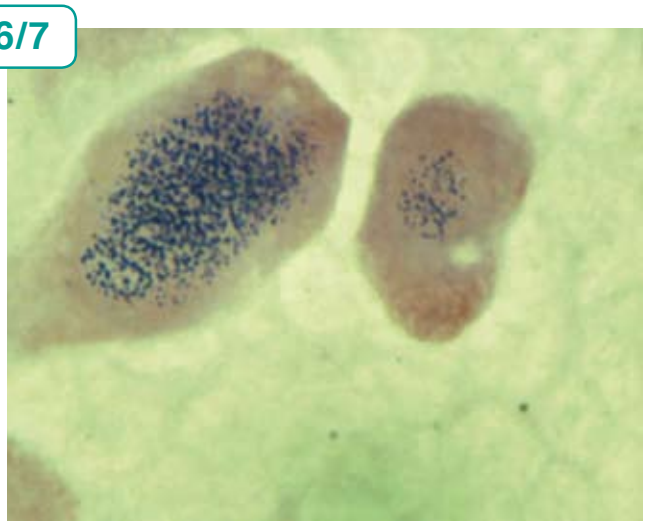

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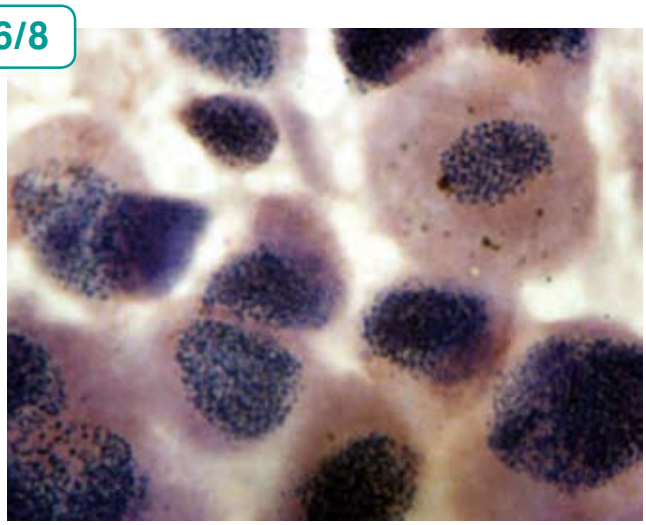

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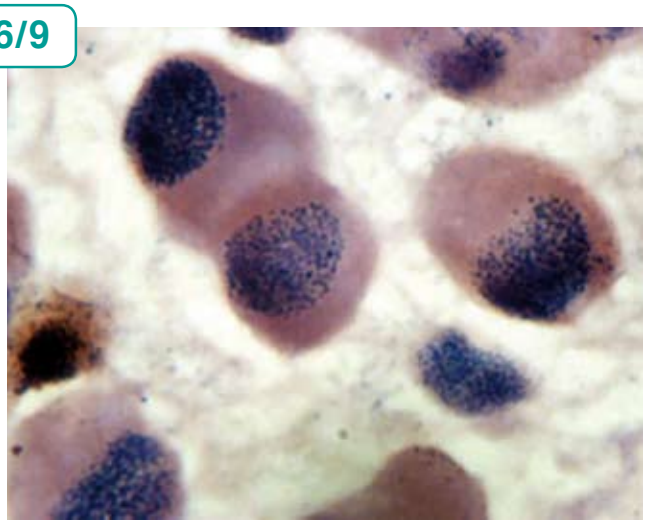

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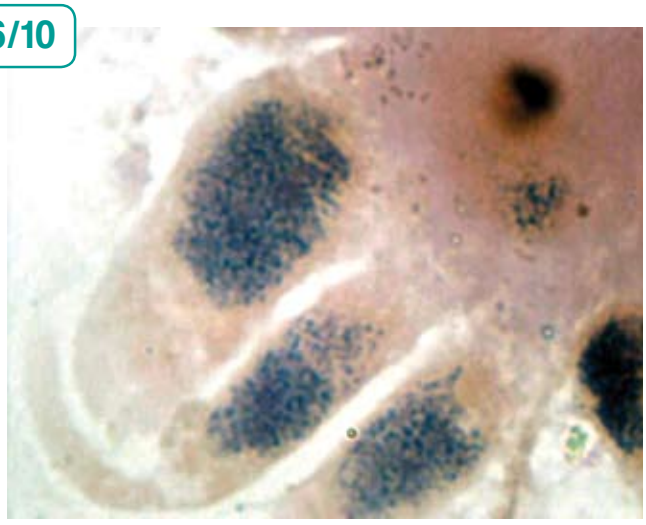

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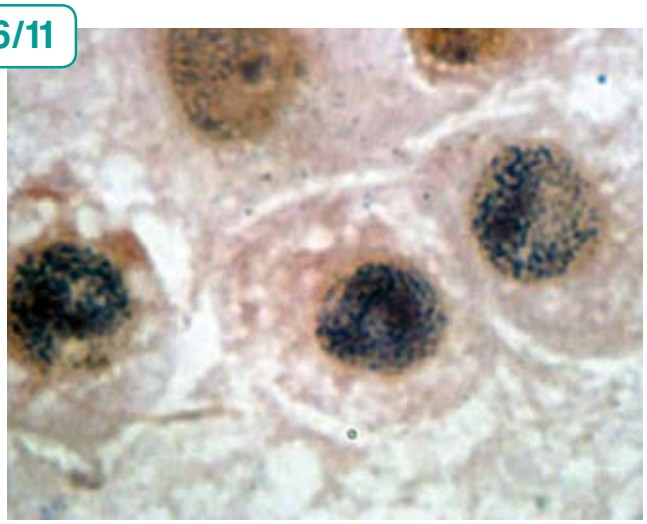

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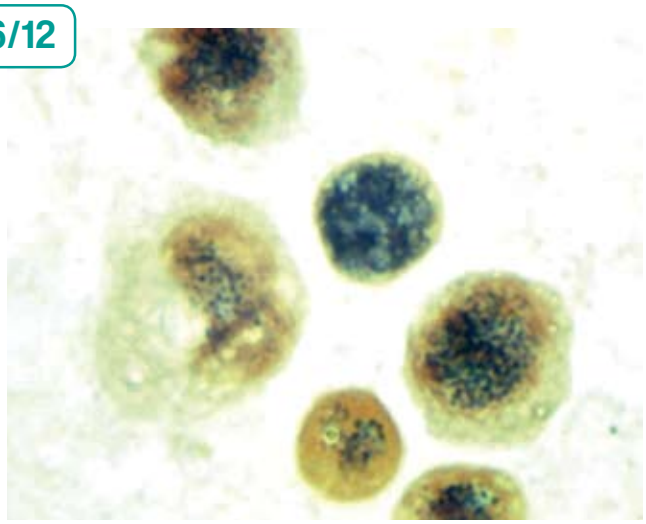
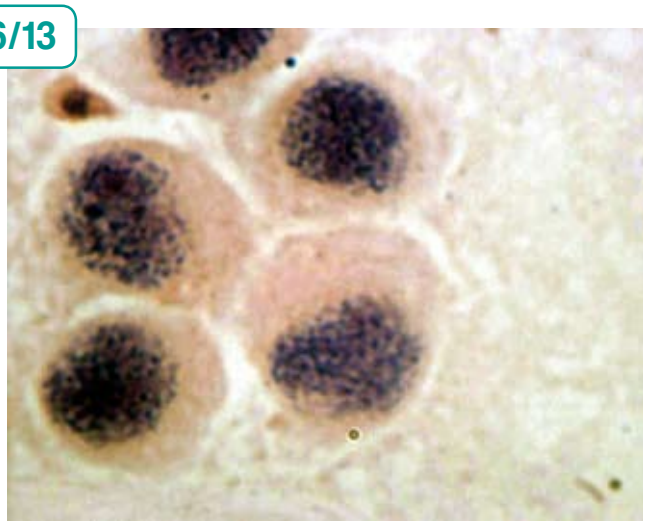


6/13



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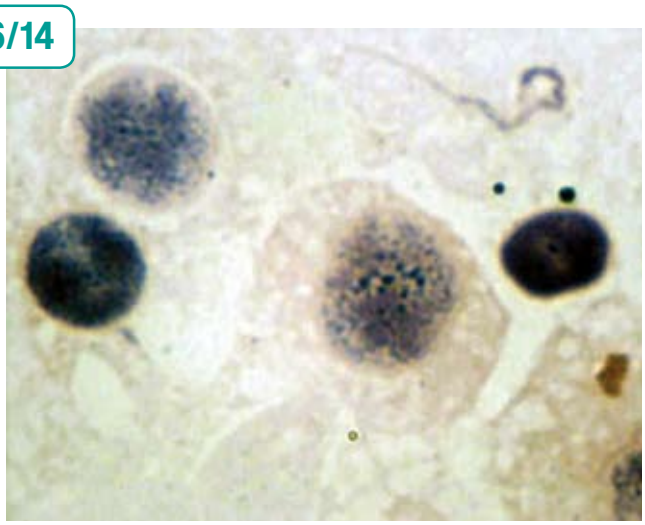

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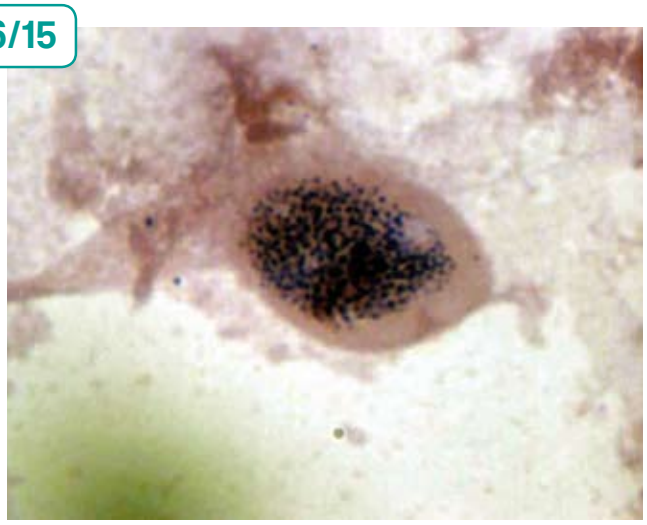

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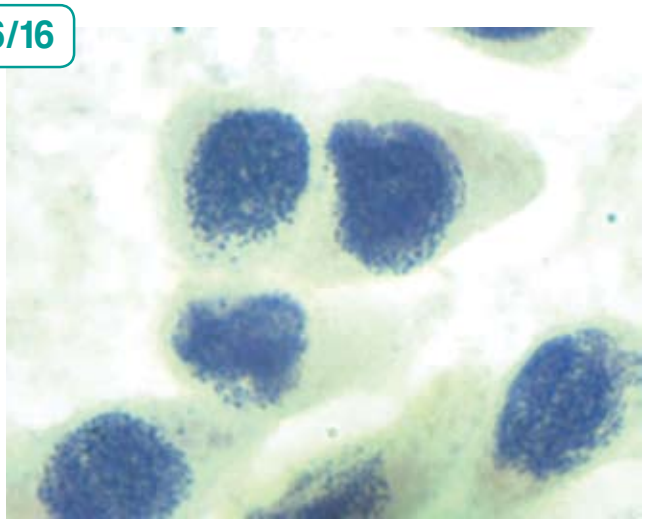

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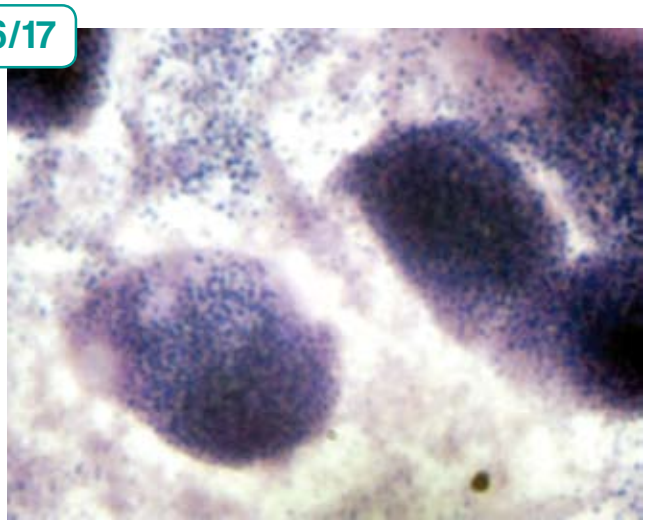

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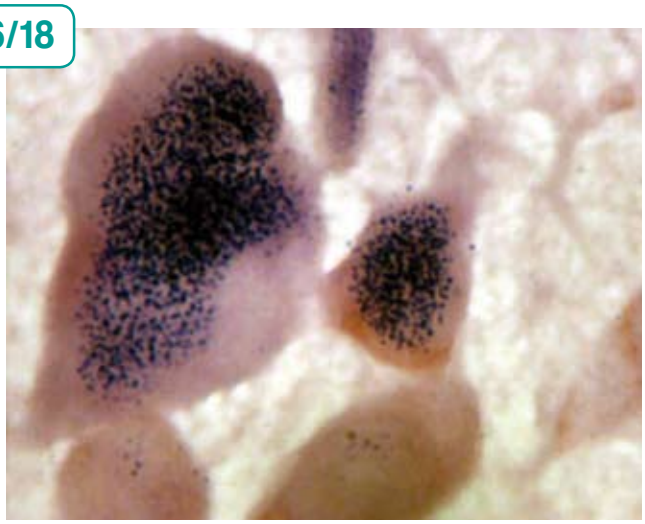

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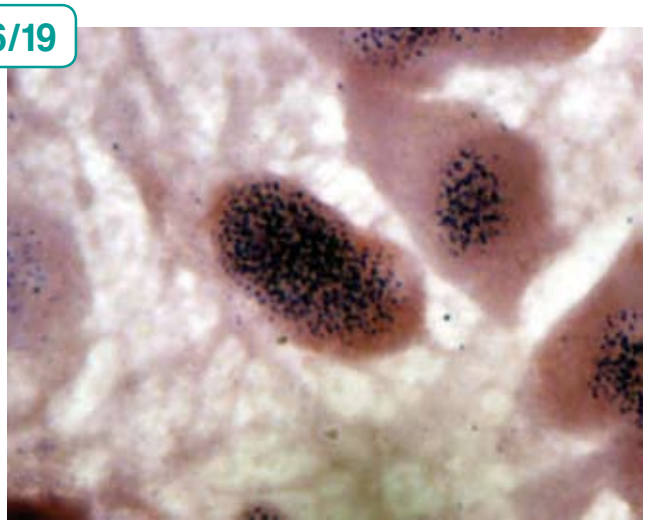

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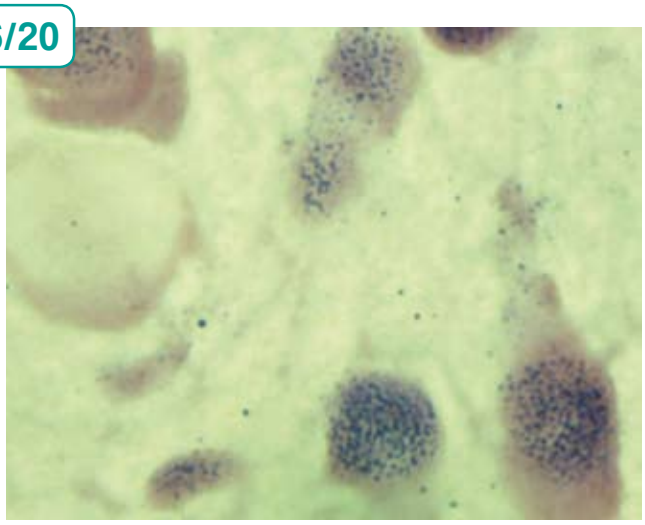

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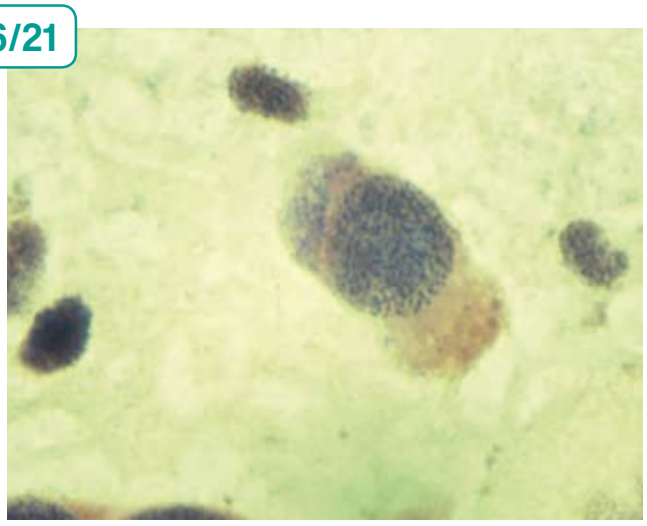

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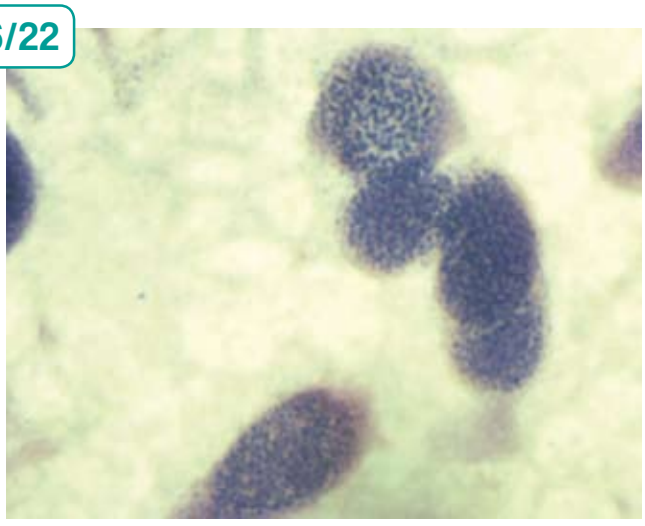

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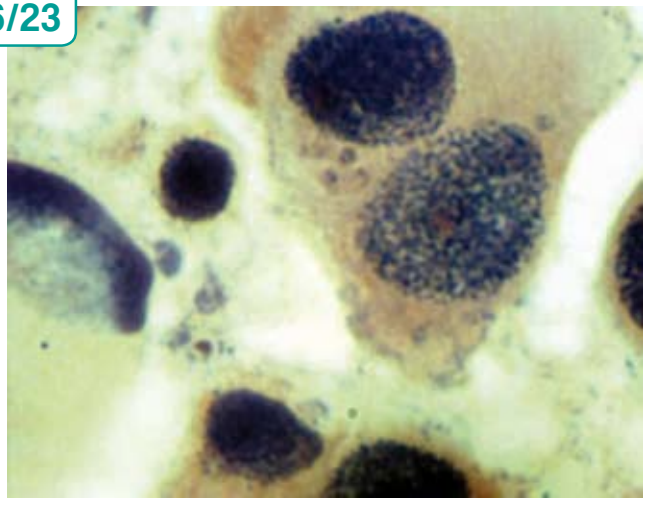

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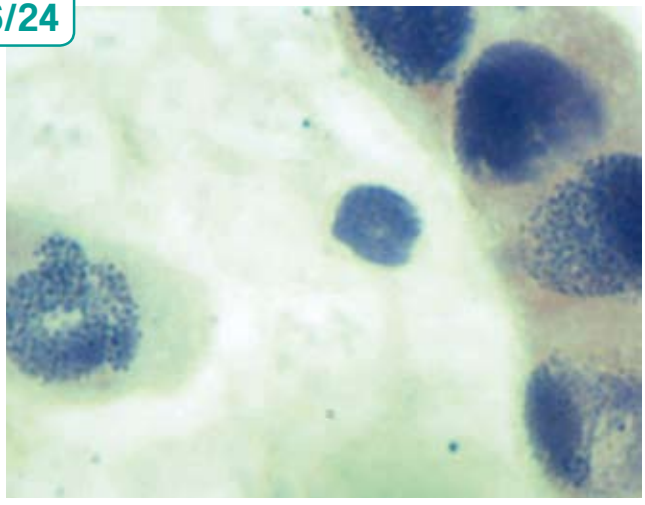

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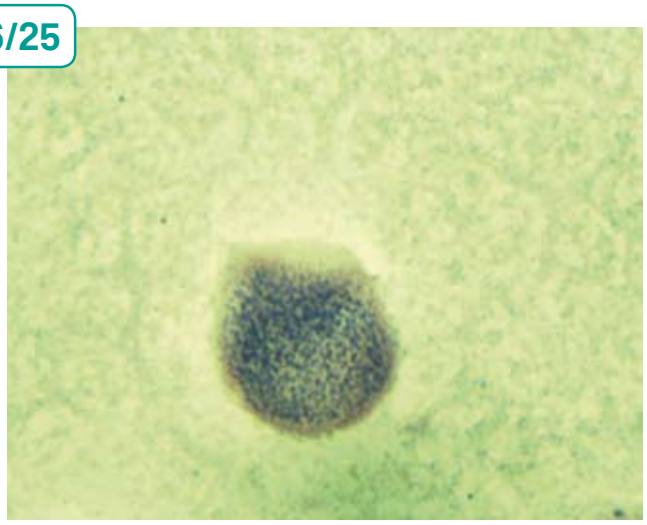
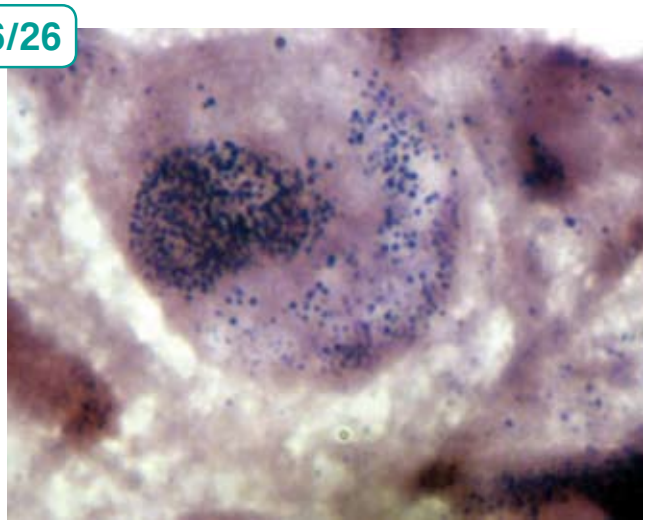


➔ **Tumor specimens;** average-size of Gram+ cocci outsider cell nuclei, show here in cytoplasm or extracellular space, are larger than those located inside nuclei (×2000) (Figures **6/26** to **6/28**)

6/26



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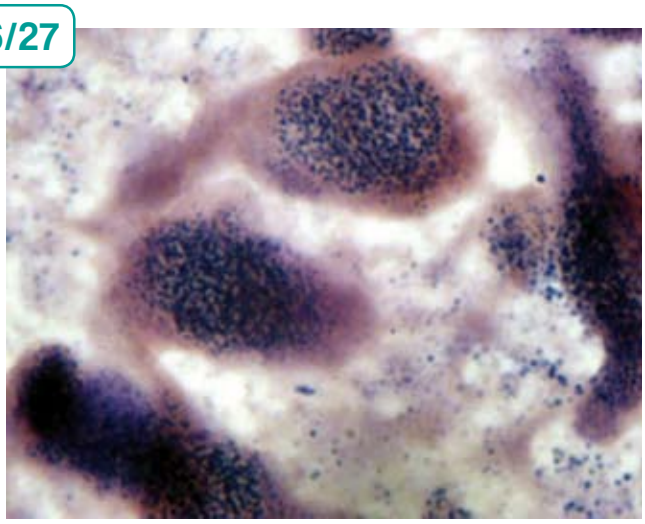
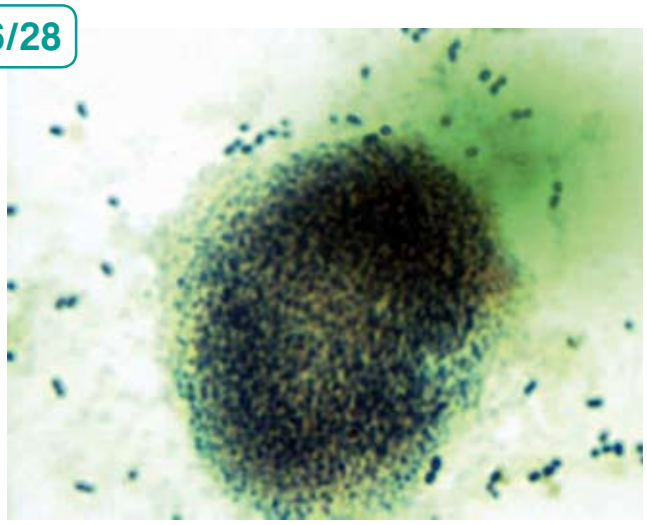
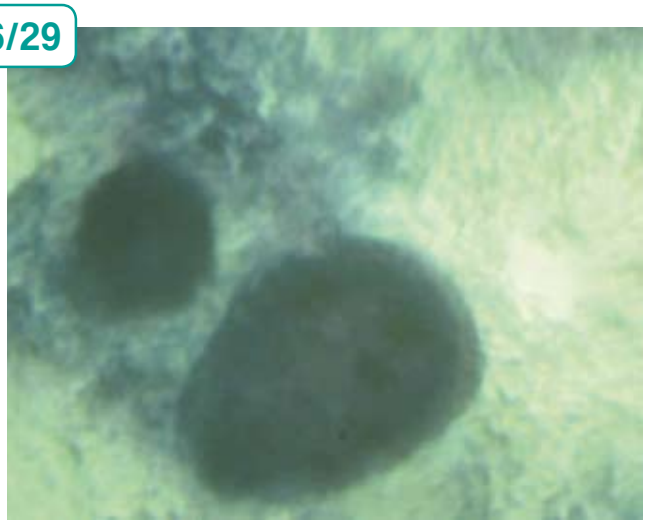


6/28



→ **Benign cells from normal lung** (Figures **6/29** to **6/36**)

6/29



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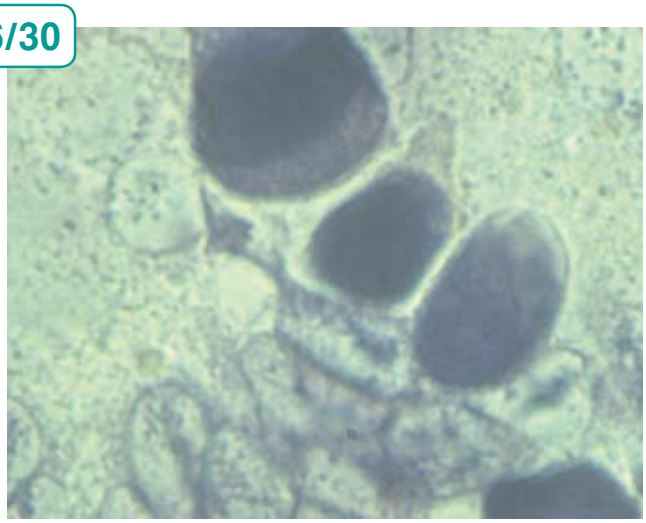
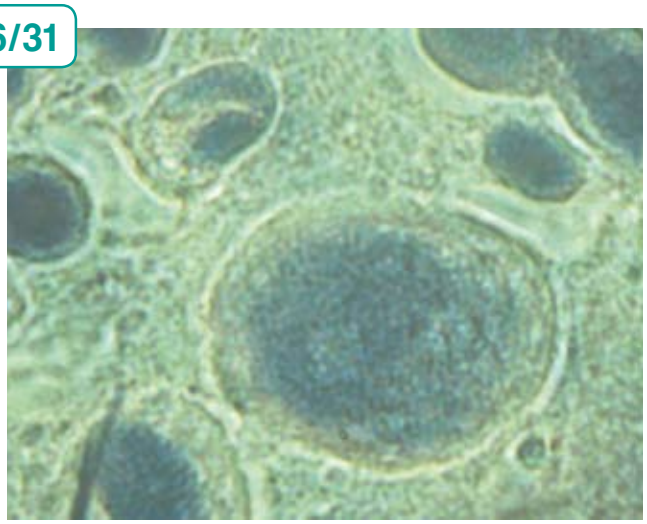
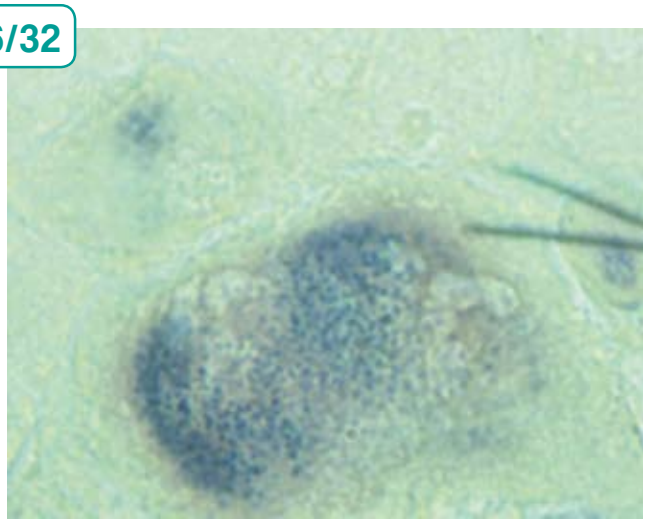


Most of bening cells are homogeneus stained with Gram (×2000)

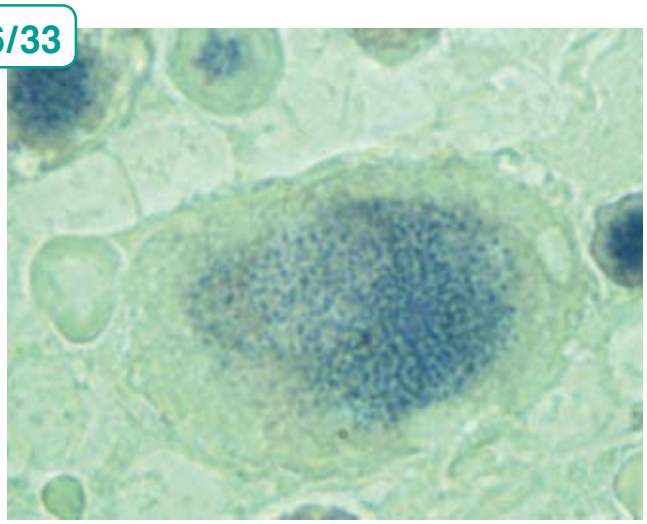
6/31



6/32



6/33



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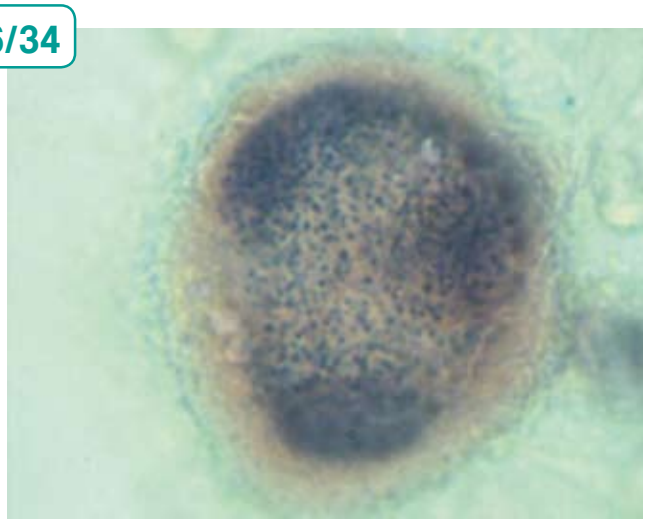

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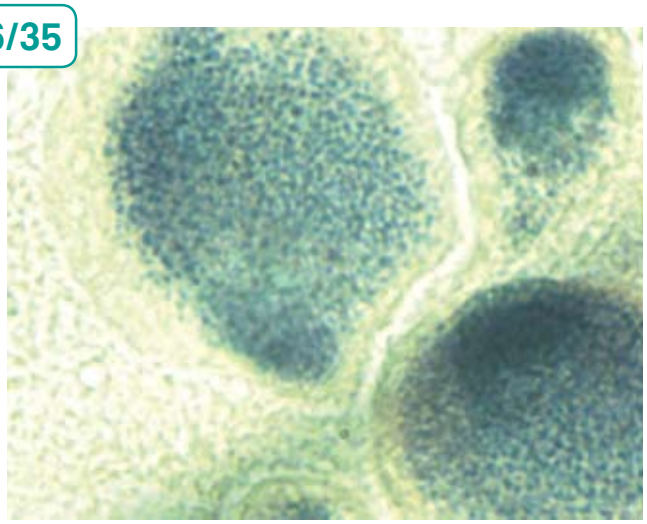
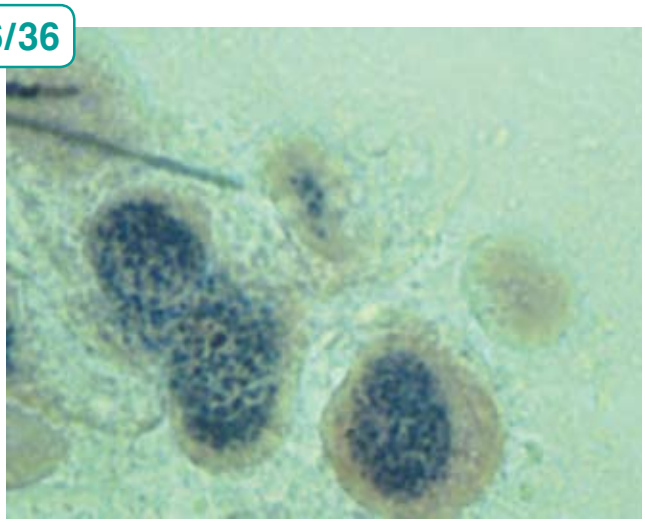


6/36



Figures 6/31 to 6/36:
Examples of non-tumoral cells shown intracellular Gram + cocci

CHAPTER 7

PRESENCE OF INTRACELLULAR GRAM-POSITIVE COCCI IN LESS FREQUENT TUMORS: SARCOMAS, LYMPHOMAS, LARGE-CELL NEUROENDOCRINE AND METASTATIC CARCINOMAS

1. Introduction

So far, we found intracellular cocci in common epithelial tumors such as squamous-cell and adenocarcinoma. It was important to know whether these cocci would be found in less frequent neoformations from epithelial source such as neuroendocrine carcinomas or lung metastases, or from other classes, such as sarcomas or lymphomas. The confirmation of the presence of the same intracellular cocci in this context could indicate a pathogenic way common to many cancers that link all neoplasms independently to their morphology or origin.

2. Materials and Methods

Using the methods described in Chapters 3, 4 and 5, specimens from malignant neoformations and non-tumor lung cells were processed, immediately after surgery. Eight lung tumors were studied: four large-cell neuroendocrine carcinomas, two pleomorphic sarcomas, one metastatic renal adenocarcinoma, and one primary pulmonary B lymphoma. Four were stained only with lacto-orcein: two large-cell neuroendocrine tumors (cases 8 and 22), one lymphoma (case 16), and one sarcoma (case 23). The rest, two neuroendocrine neoplasms (cases 35 and 39), one sarcoma (case 45), and one metastatic renal carcinoma (case 48, which had been removed by a nephrectomy 10 years before), were stained with lacto-orcein and Gram (Table 2/1).

3. Results

All tumor samples contained cocci, those stained with lacto-orcein alone and those stained with lacto-orcein and Gram. With respect to the lacto-orcein stains, pleomorphic sarcoma cells showed abundant and clear intracellular cocci (Figures 7/1 to 7/6). Smears from diffuse B lymphoma also presented numerous cocci (Figures 7/10 to 7/18).

Two neuroendocrine tumors had many cocci, which were clearly observed because, generally, cells from this type of tumor are larger than other epithelial malignant cells (Figures 7/19 to 7/29). The same morphologies were detected on cells of metastatic adenocarcinoma (Figures 7/37 and 7/38).

With respect to Gram stains, pleomorphic sarcoma cells (Figures 7/7 to 7/9), large-cell endocrine tumor cells (Figures 7/30 to 7/36), and metastatic adenocarcinomas cells (Figures 7/39 to 7/45), had many Gram-positive cocci similar to those found in squamous or adenocarcinoma cells. There was one case of inflammatory pseudotumor, but it was not included here because it is not considered a true tumor. It contained large, anomalous cocci and it was considered one of the false-positive results.

4. Discussion

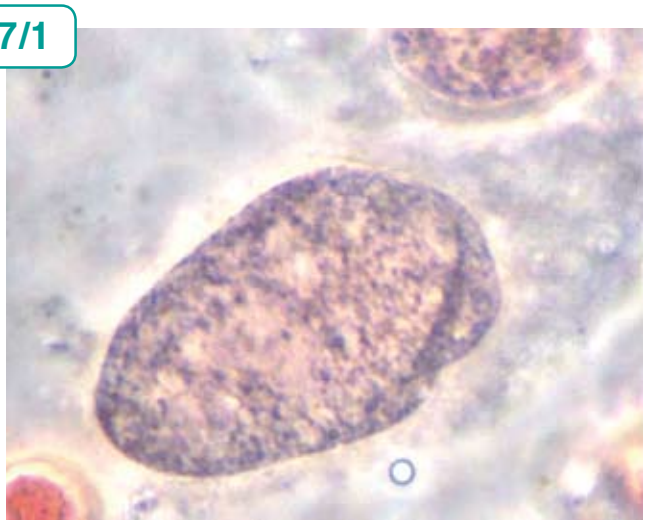
The presence of intracellular cocci was seen also in less frequent epithelial tumors and those derived from connective or lymphatic tissues. Although these cocci were identified in several hundreds of such cells, these observations must be confirmed with a bigger number of cases. These results strongly support the notion that among the different types of tumors, and among the different anatomical origins of these tumors, there is a common pathogenic trait. This pathogenic trait is these Gram-staining cocci that live inside malignant cells and which belong to a structure different to that of the host cells. This repeated unique morphology present in such different classes of tumors must have an important pathological significance that could explain cancer characteristics such as growth and spread, virtually independent from the family, the anatomical origin, or the morphology of the malignancies. Thus, these data reinforce the notion that intranuclear cocci could be a pathognomonic finding of tumors.

Cases numbers 69 and 71 (Chapter 10), will be observed during the year 2012, and both will be pulmonary metastases of gastric and endometrial adenocarcinomas respectively containing plenty of intracellular cocci (see Chapter 10, Figures 10/2 and 10/7). Thus, samples of the 3 of the most important sources of lung metastases, kidney, digestive and genital tracts, present the same cocci picture.

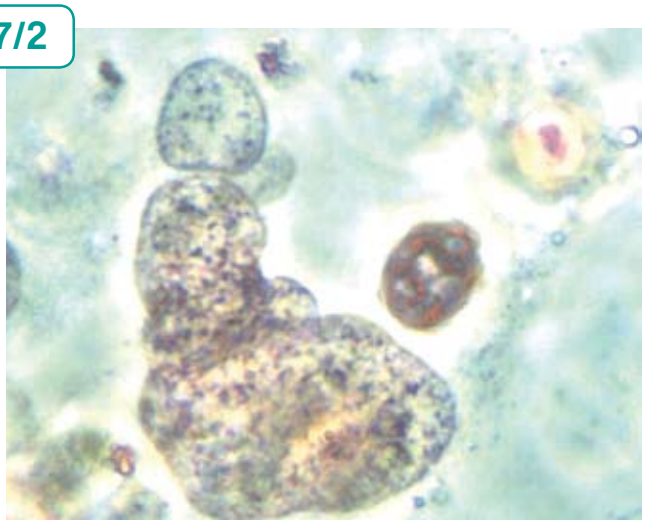
Pleomorphic sarcoma intracellular cocci. Lacto-orcein staining (×2000)

FIGURES 7/1 TO 7/6

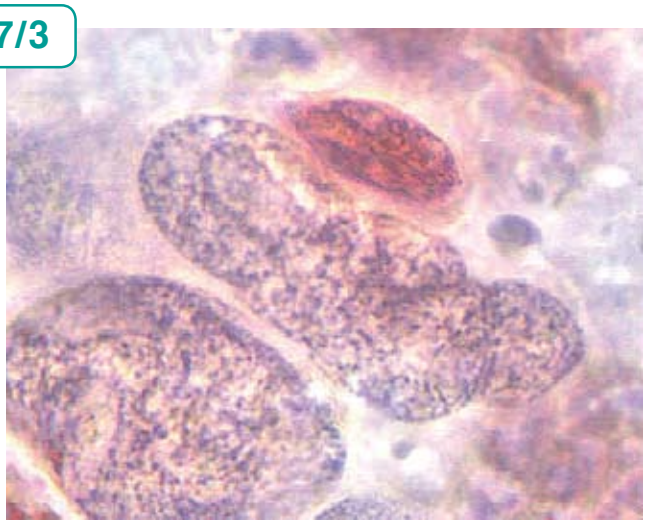
7/1



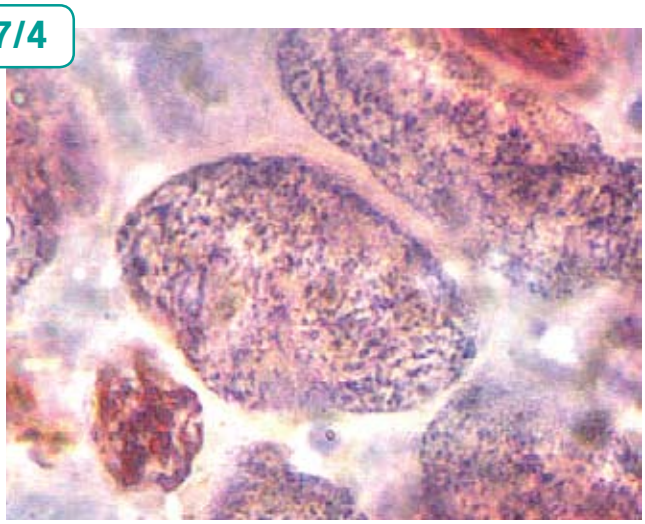
7/2



7/3



7/4



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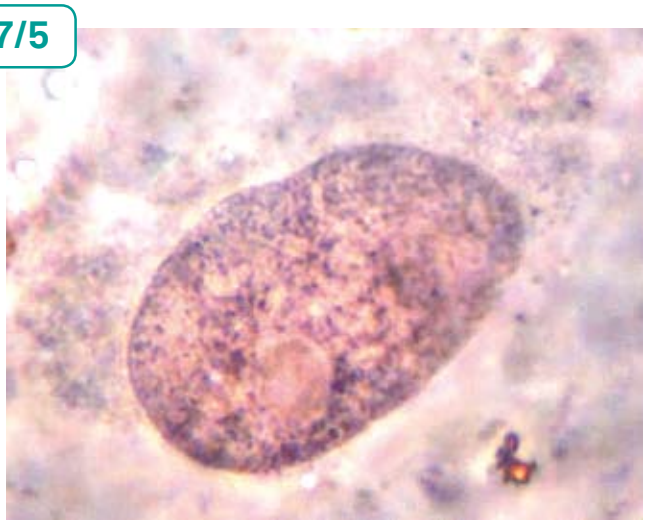

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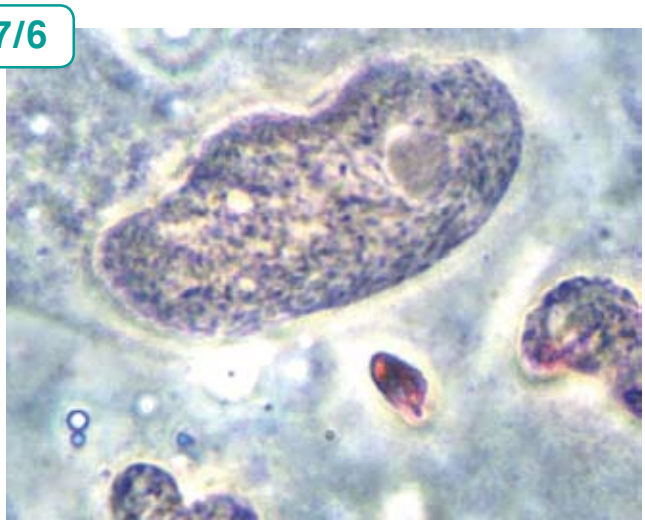
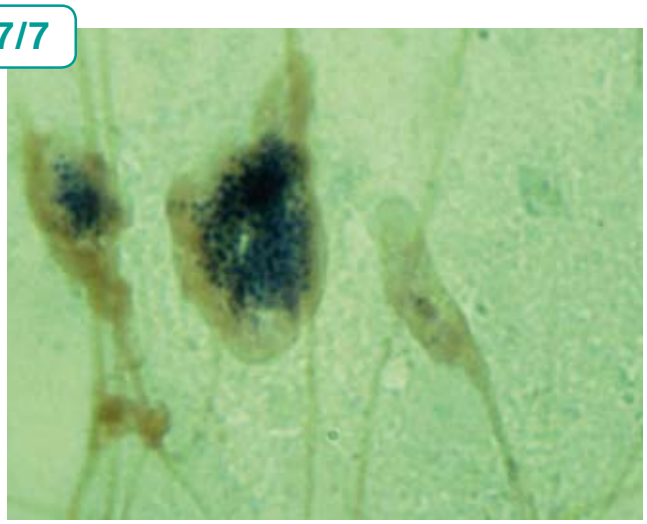


Pleomorphic sarcoma. Gram staining (×2000)

FIGURES 7/7 TO 7/9

7/7



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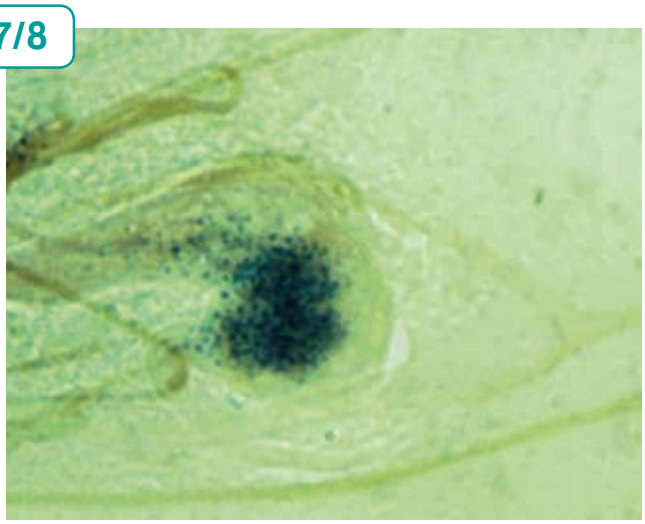

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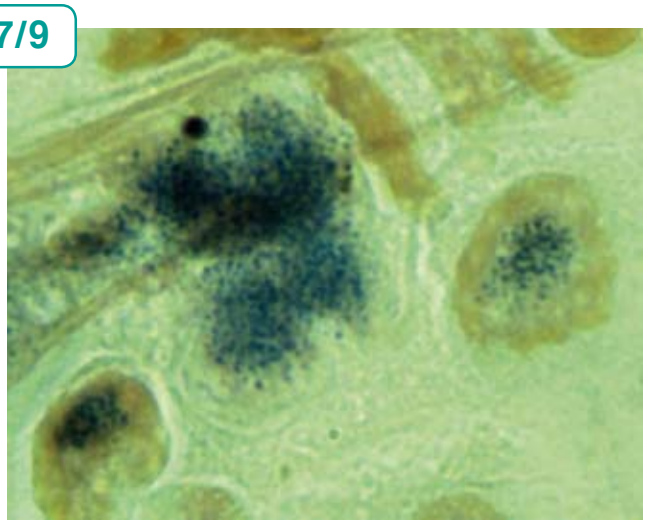
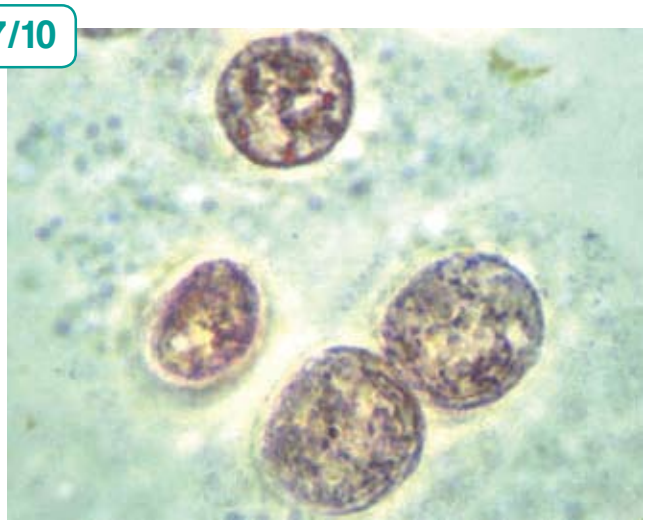


Pulmonary diffuse B lymphoma cocci inside cells. Lacto-orcein staining (×2000)

FIGURES 7/10 TO 7/18

7/10



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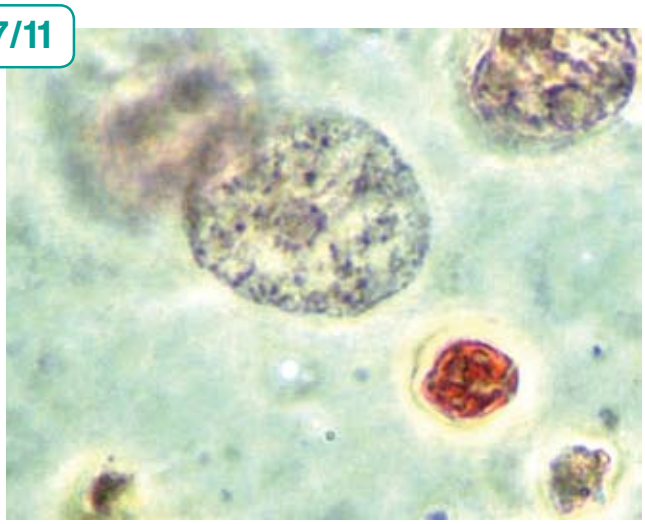

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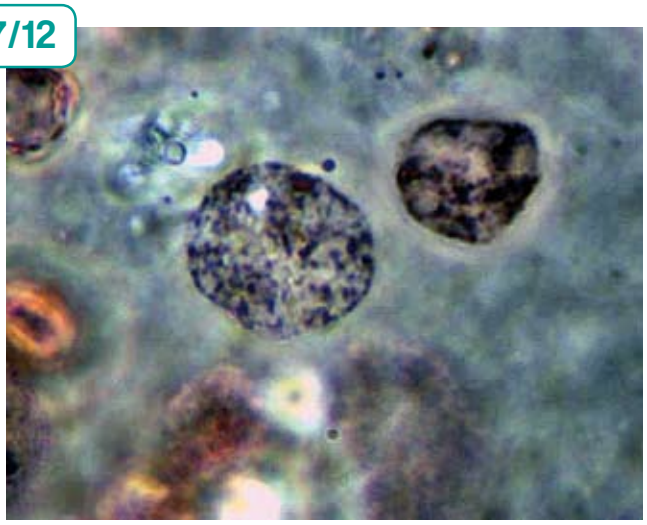

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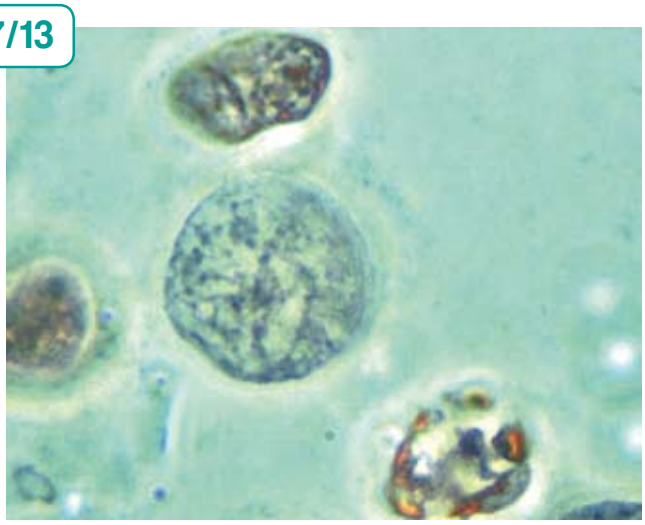

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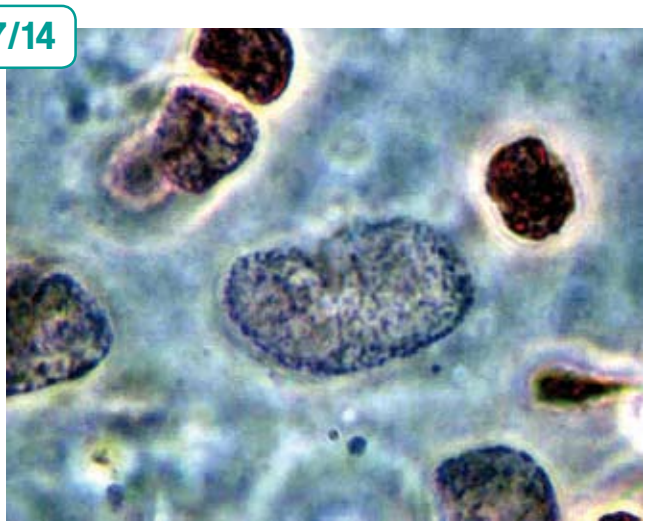

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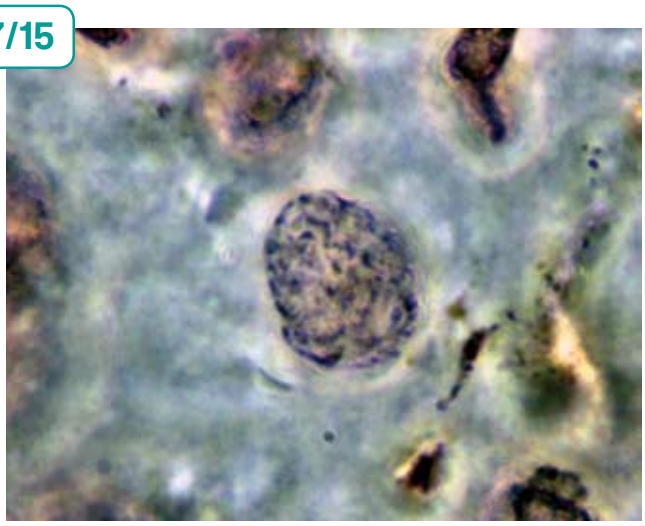

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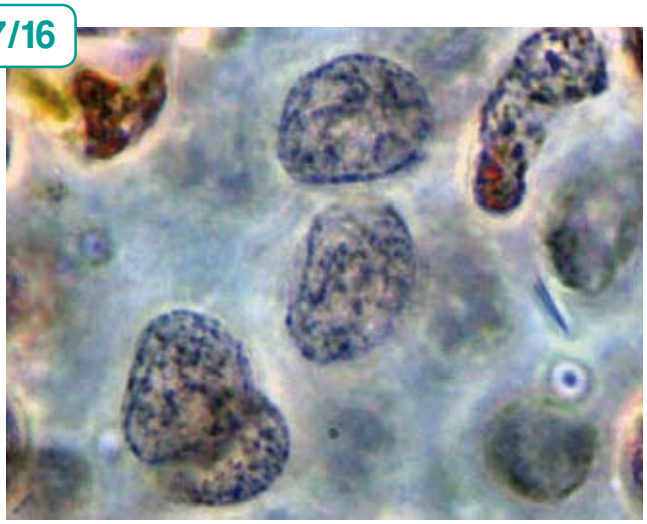

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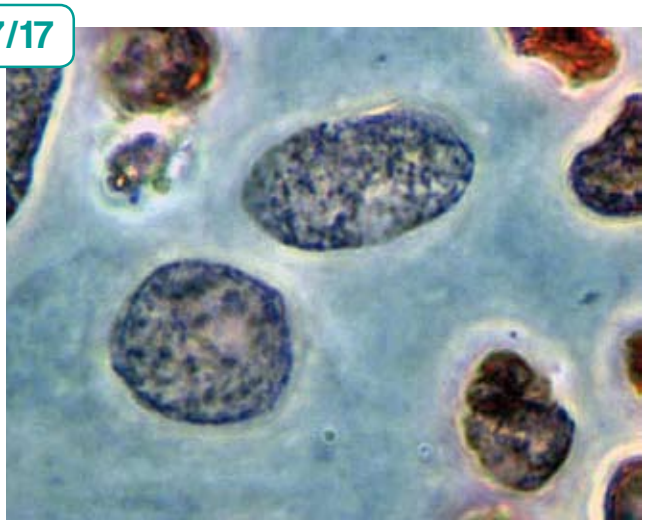

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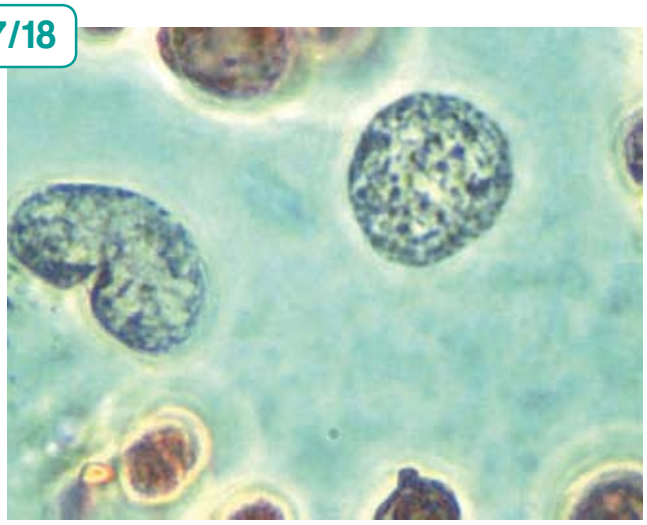
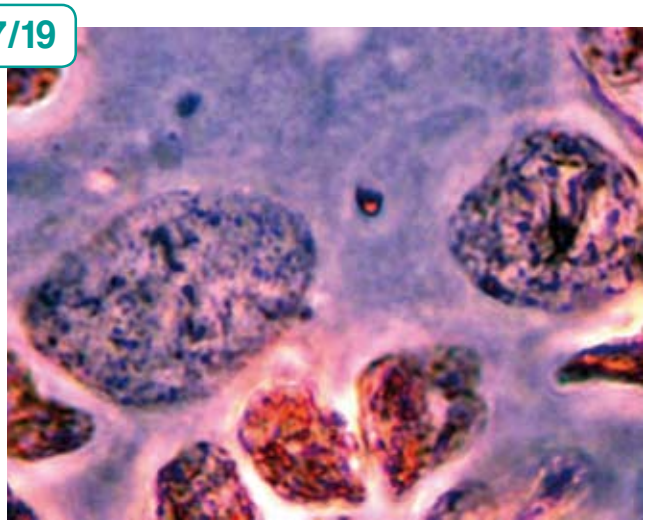


Large-cell neuroendocrine carcinoma: intracellular cocci.
Lacto-orcein staining (×2000).

FIGURES 7/19 TO 7/29

7/19



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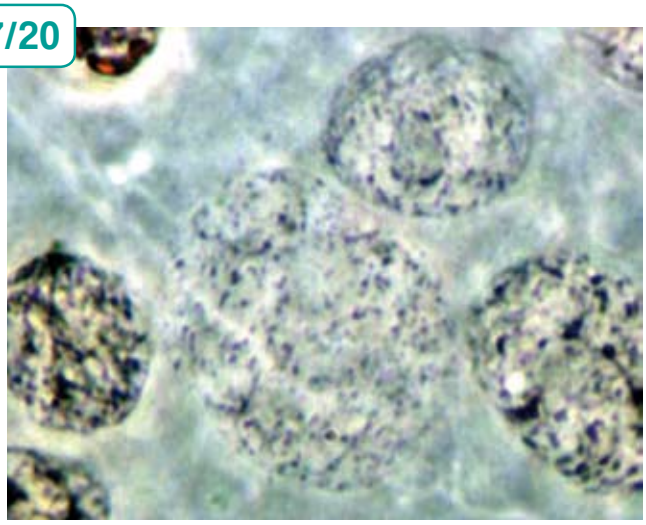

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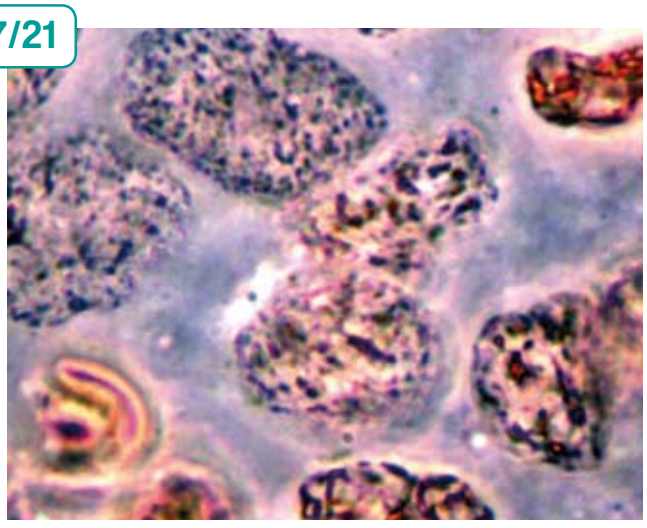

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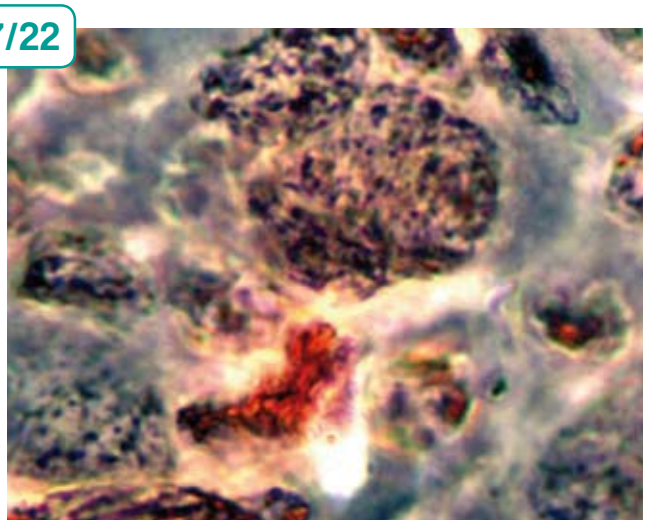

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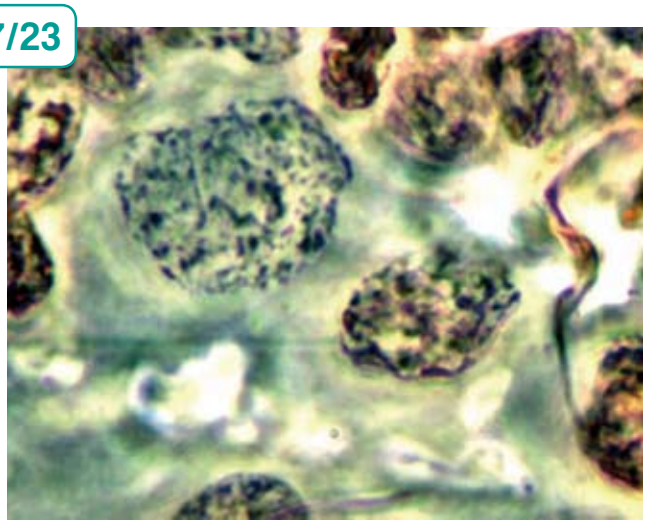

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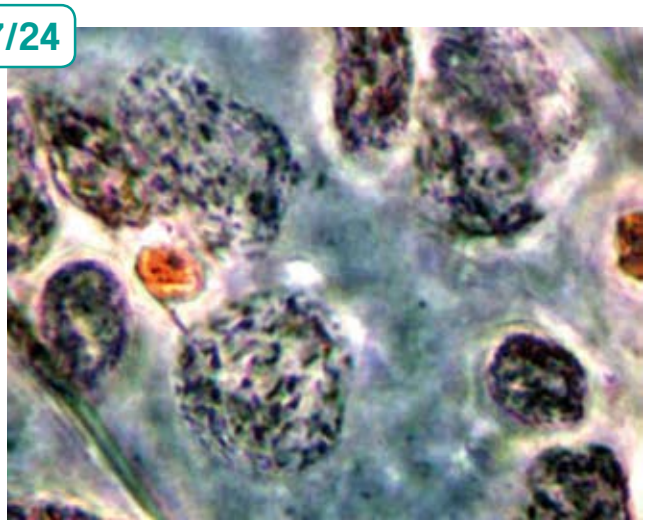

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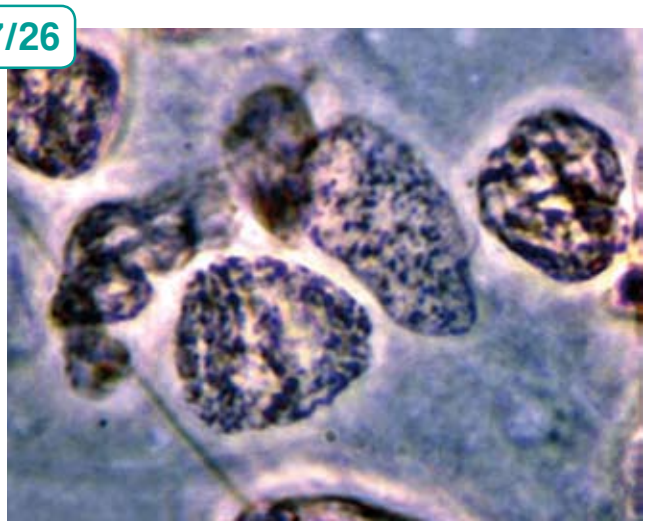
7/26



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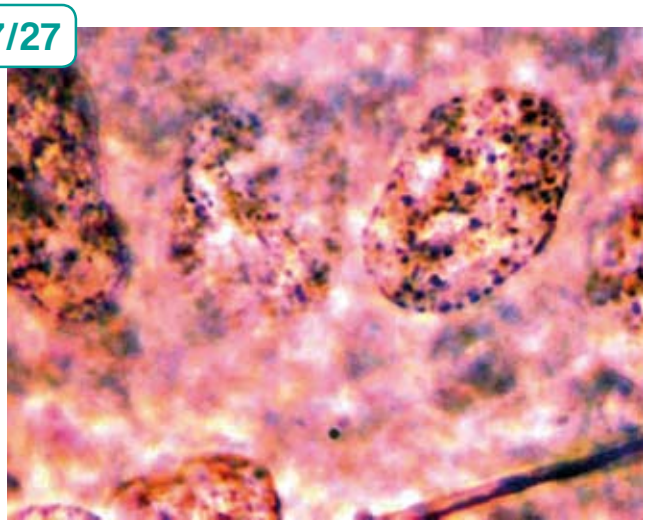

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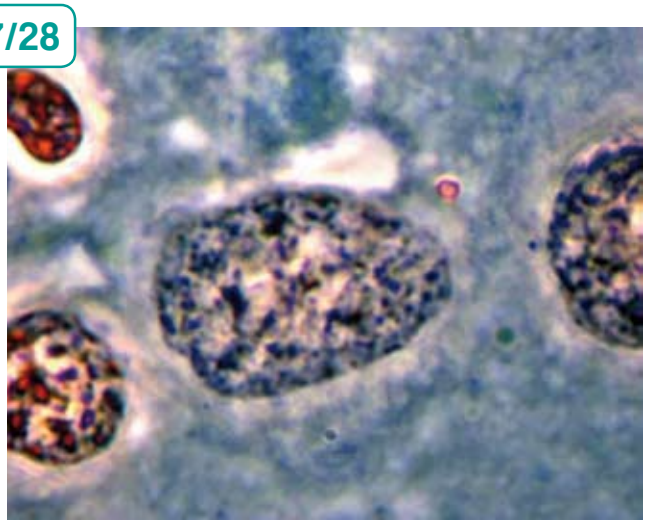

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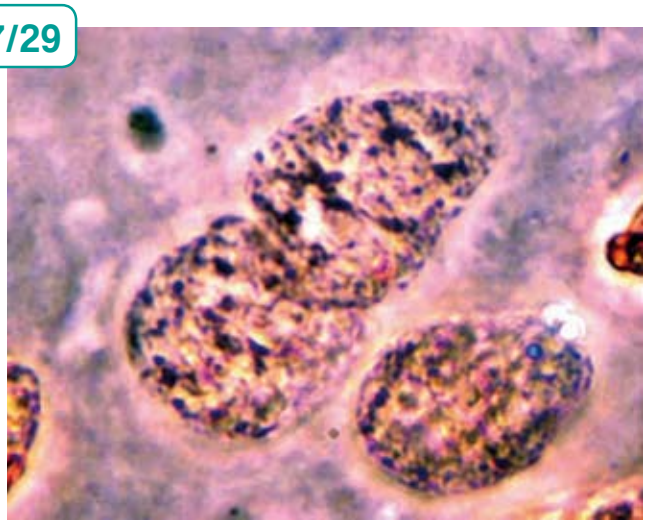
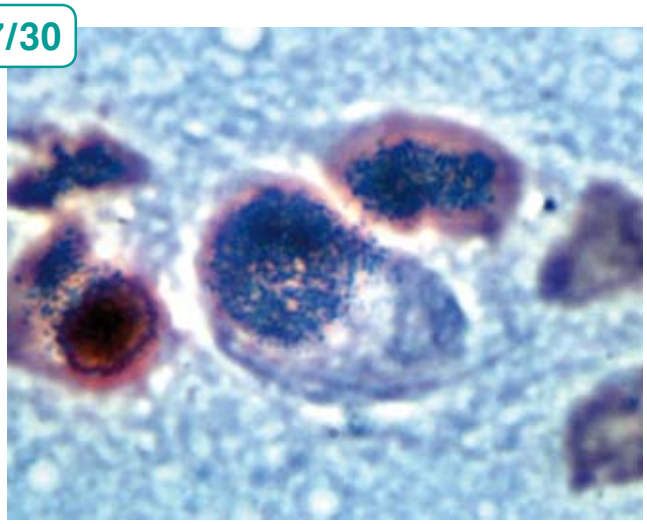


Large-cell neuroendocrine carcinoma: intracellular cocci. Gram staining (×2000)

FIGURES **7/30** TO **7/36**

7/30



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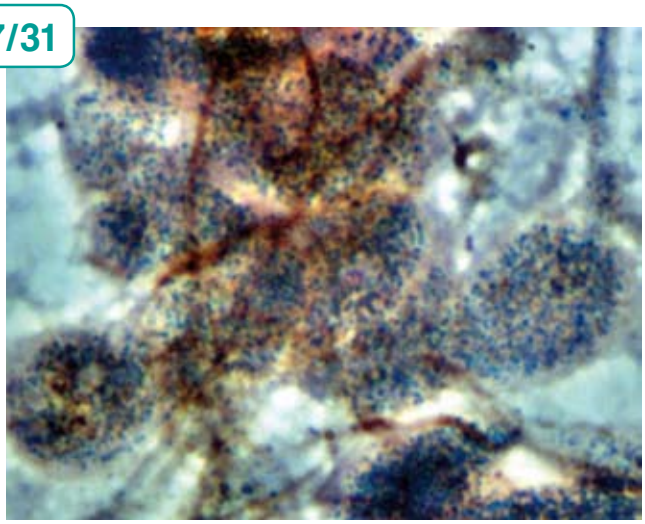

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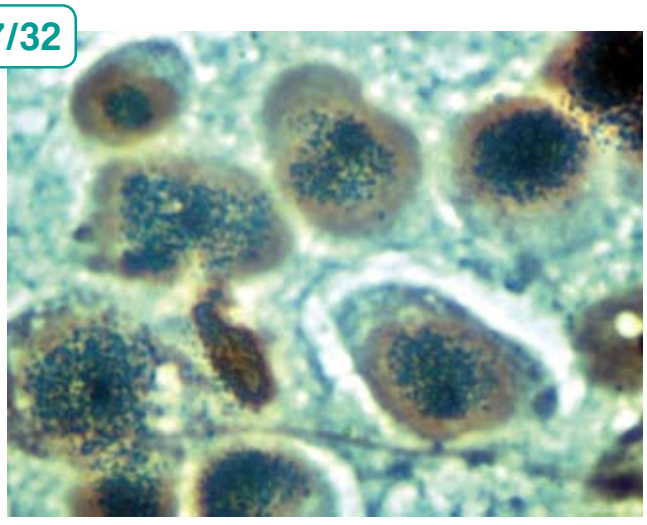

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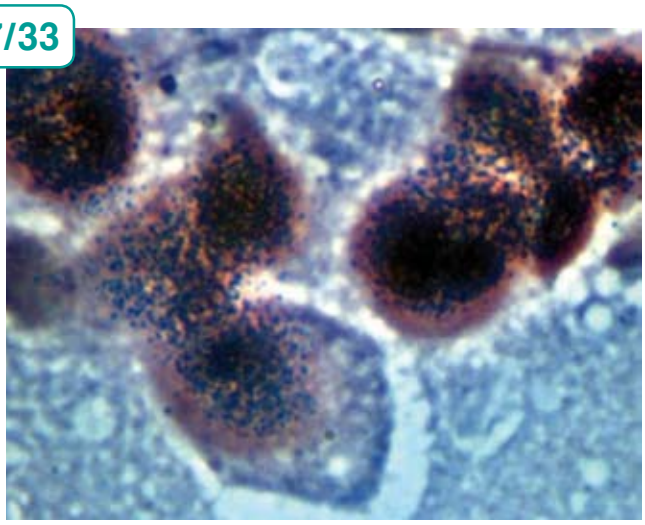

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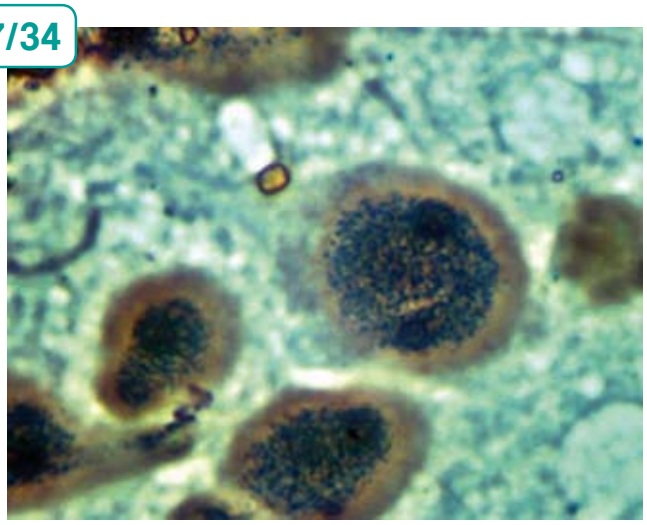

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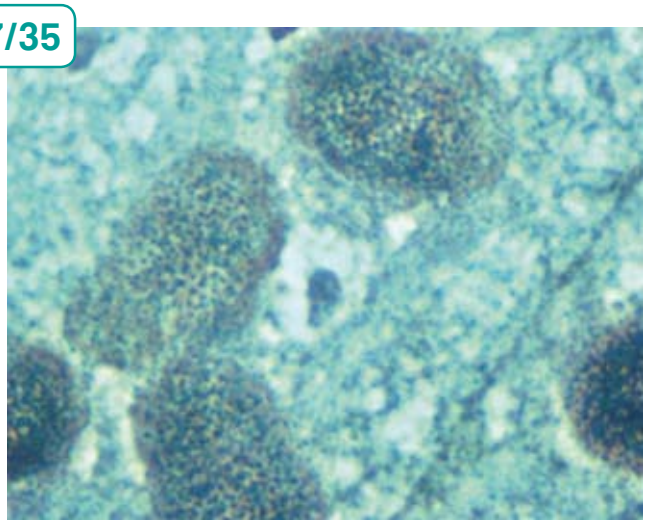

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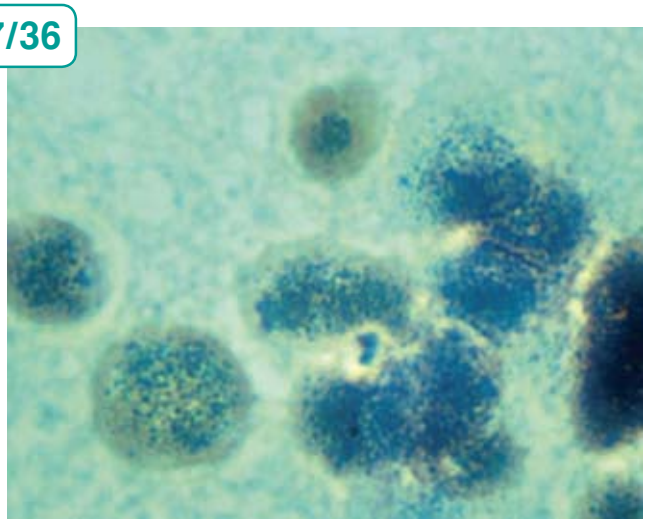
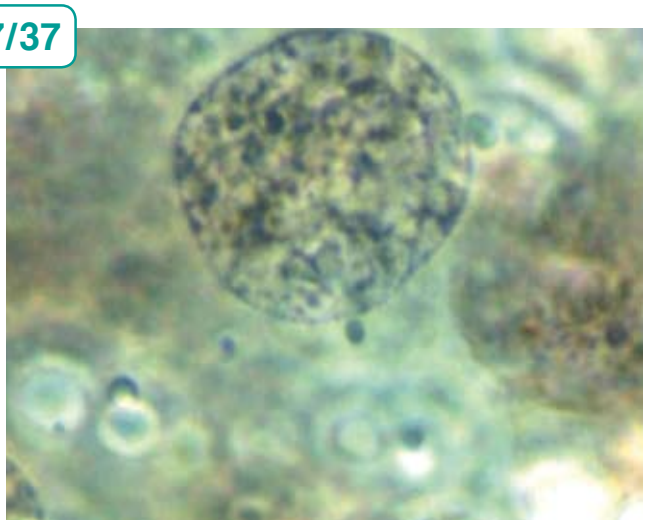


Metastatic renal carcinoma with intracellular Gram + cocci.
Lacto-orcein staining (×2000)

FIGURES 7/37 AND 7/38

7/37



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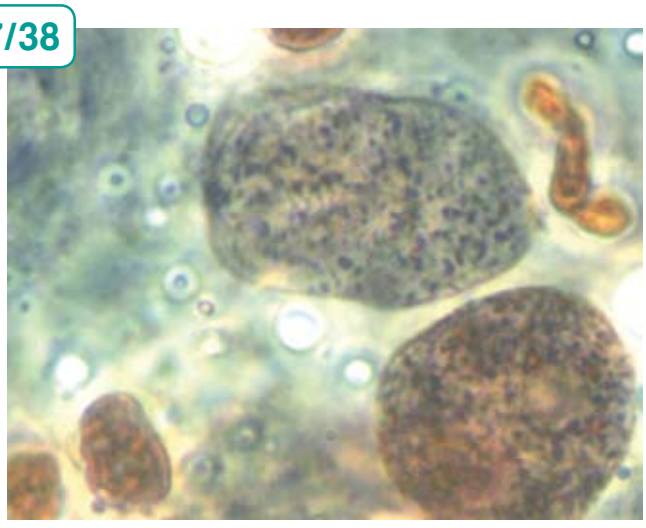
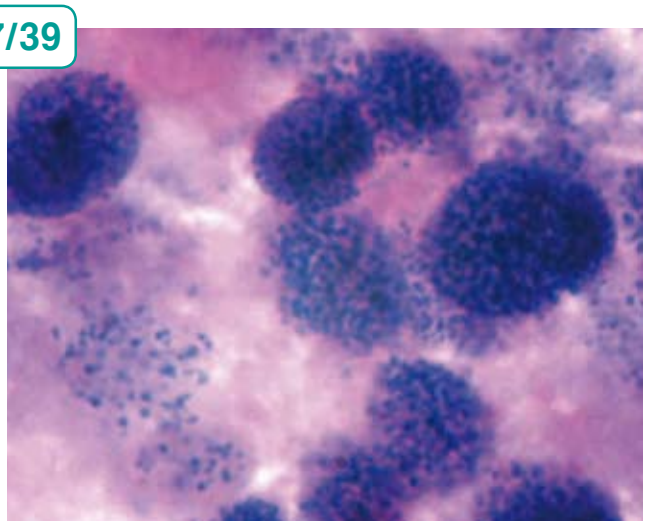


Metastatic renal carcinoma with intracellular Gram + cocci.
Gram staining (×2000)

FIGURES 7/39 TO 7/45

7/39



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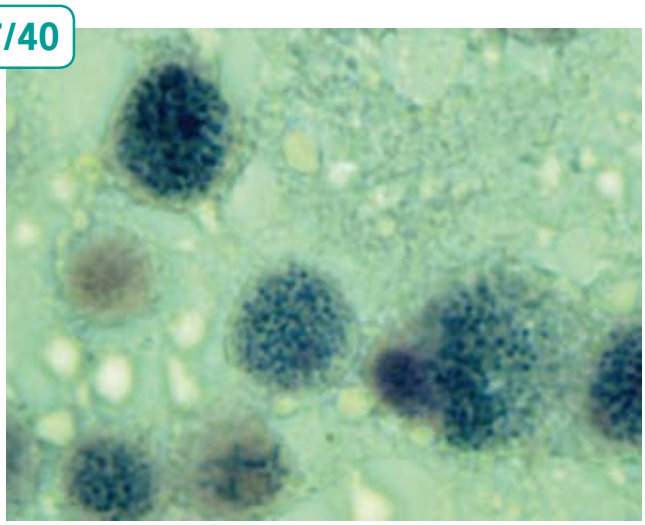

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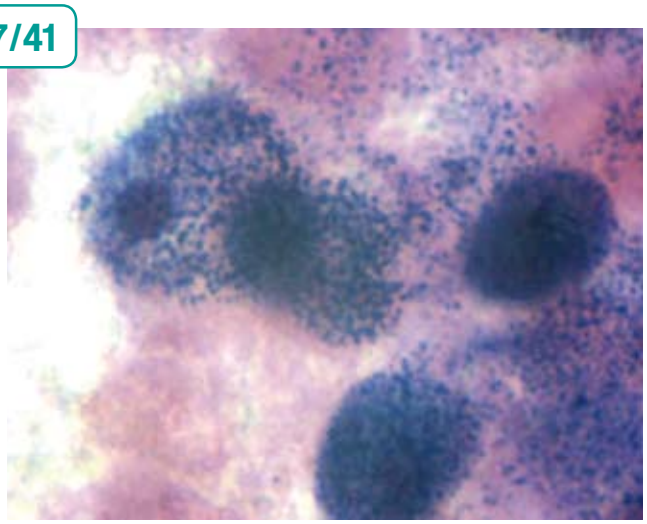

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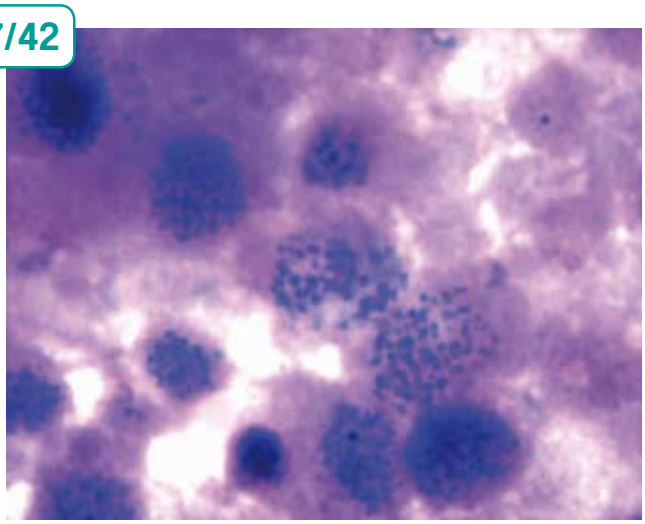

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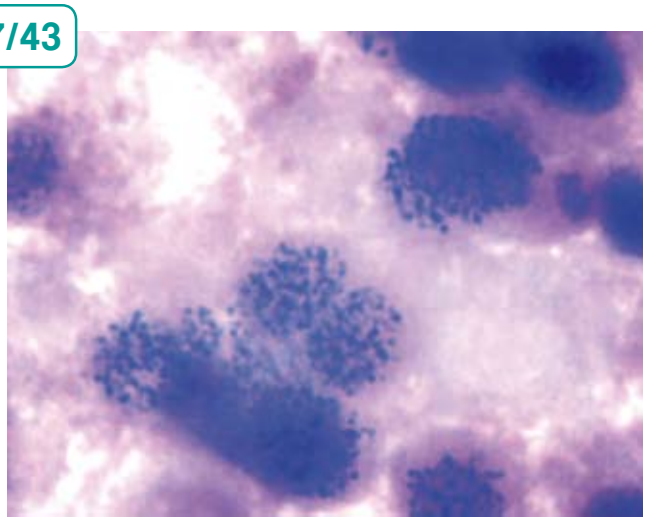

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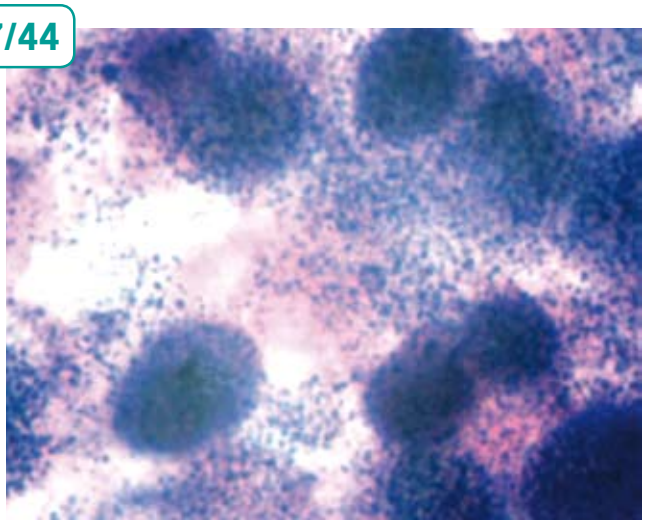

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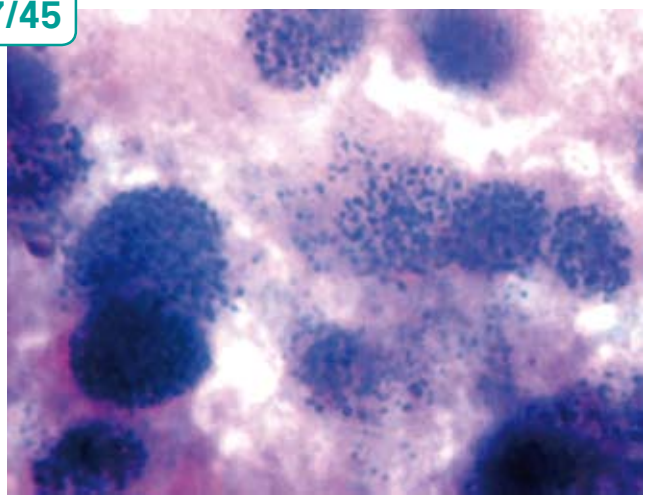


CHAPTER 8

FLUORESCENT STAINING: LIVING BACTERIA INSIDE TUMOR CELLS

1. Introduction

The genesis of malignant tumors remains unknown. The finding of endosymbionts filling these tumor cells in all cases studied enlightens the aetiology of cancer. The mechanism through which these Gram-positive cocci cause a massive production and widespread of host cells must be studied. This can be done by marking cellular nuclei and endosymbionts, both main protagonists of this process. Staining with Syto 9 plus Propidium iodide allowed us to detect nucleic acids with intact or damaged cell membranes of tumors and bacteria, and their interaction.

2. Material and methods

Samples of successive tumors and neighbouring normal lung from 20 patients were obtained and stained within 3 min to 96 h after surgery procedure (cases 49–68; Table 8/1). The samples were processed in a sterilized cabin (Telstar bio II A/P) with the same conditions as those described in Chapter 3. They were cut into small fragments and were diluted in similar volume (1:1, v/v) of sterilized water. One or two drops of the diluted mixture were placed on sterilized microscope slides, and smears were covered with a Syto 9 plus Propidium iodide stain mix (live/dead BacLight Bacterial viability kit, Molecular Probes L-13152) prepared as recommended by the manufacturer (1:1, v/v). The Syto 9 dyes nucleic acids, giving them a green fluorescent light, independently of the state of the membranes (intact or damaged). Propidium iodide only enters the cells with damaged membranes, and gives the nucleic acids a red fluorescent light, causing a reduction in the Syto 9 stain because of its higher affinity for nucleic acids. After leaving the staining for a least 10 min in the dark, the samples were observed under a Bio-Rad MRC 600 laser confocal microscope at 488 nm and 568 nm excitation and 530 nm (green) or 630 nm (red) emission. Both images were mixed with the Confocal Assistant version 4.02 programme (Todd Clark Bridge,1994-96, freeware programme distributed by Bio-Rad Laboratories) in order to make up the final dual-colour image.[1] Smears were also stained with Gram staining, following the described methods.[2]

4068 cells were useful to study, (identifiable cells under fluorescent stain): 3848 were living cells, of which 3285 were obtained from tumors and 563 from non-tumoral resected lung. 220 dead tumor cells were stained within 6 min after surgical procedure and were also included in the study, because the time spent after surgery manipulation was very short, and cells were considered to have died before their isolation from the human body. Data were analyzed statistically (Chapter 2).

3. Results

Samples from 20 successive thoracotomies were obtained from lung cancer tissue and neighboring healthy lung tissue. 12 (60%) were adenocarcinomas and the other eight corresponded to squamous-cell cancers. Characteristics of these samples such as staging or type of surgery can be seen in Tables 8/1 and 8/2. 17 (85%) came from men and three (15%) from women. Individuals had a mean age of 65.5 years (1SD 10).

60 dead benign cells were measured only for size comparison with 563 living benign cells.

1 Fernández M , Sánchez J . *Nuclease activities and cell death proceses associated with the development of surface cultures of Steptromices antibioticus ETH 7451*. Microbiol 2002; 148: 405-12.

2 Gardner P, Provine Ht. *Manual of acute bacterial infections. Early diagnosis and treatment* . 1st ed. Boston. Little Brown Co (inc), 1975: 168.

The mean of maximum diameter of living benign cells was 10.6 µm (1SD 4.6) and did not differ statistically with that of the dead benign cells. Living benign cells had a homogeneous aspect and showed green fluorescent light without any other characteristic, except, in four cases, bacteria were observed scarcely located inside the cells (Figures 8/2 to 8/16).

Mean of maximum diameter of 3285 living tumor cells was 11.2 µm (1SD 6.6), and did not differ statistically from that of living benign cells, but was larger than that of dead benign cells ($p = 0.02$). The mean diameter of dead tumor cells was 18.0 µm (1SD 6.8) and it was larger than that of all other cells ($p = 0.000$).

The total cancer cells useful to study were 3505 (3285 living and 220 deaths) and they were classified according to their fluorescent characteristics into five types.

1313 (37.5%) of these cells were round shaped and had two main characteristics: a strong homogeneous green fluorescence with no internal structure observable and a small size, with a mean length of 6.9 µm (1SD 4.0). These cells were significantly smaller than the other cells, $p = 0.000$. These cells will be referred to as living small dense cells from now on.

Nearly half of all cells were large inhomogeneous green fluorescent cells, with two morphologies: clusters of living bacteria or big green round corpuscles with close aspect of small dense cells, but they remain inside cells. It is necessary to insist that both morphologies are inside the same cells, at the same time, but there is always a predominance of one of the forms or the other, thus it is useful for distinguishing between large cells: those with predominance of bacterial clusters or those with outstanding new nuclei.

In fact, 803 (22.9%) living large cells with viable bacterial clusters have a diameter ($\tilde{x} \pm 1SD$) of 14.9 ± 7.5 µm, twice than small dense cells ($p = 0.000$). They contain among 6 to 196 viable bacteria, with a mean (72.5 ± 72.8 bacteria per cell). The diameter of intracellular living bacteria, observed with Syto plus Propidium iodide was 1.0 ± 0.2 µm. 972 (27.7%). Viable large cells with predominance of new nuclei are 14.3 ± 5.7 µm, and they are also larger than small dense cells ($p = 0.000$) but not different to those with a predominance of bacteria. Each cell contains 2.4 ± 0.9 new nuclei, and they measure: 2.2 ± 0.8 µm in length. The theoretical number of new nuclei that can be released from cells will be the result of the percentage of new nuclei-bearing cells by the mean of new nuclei per cell. This product can be called *"new nuclei load"* with a mean value for all 20 tumors of 83.3 ± 64.9 per 100 cancer cells.

197 (5.6%) viable neoplasic cells present a weak green fluorescence, without any visible structure inside cells, and so they can be called empty living cells. Their size is 10.5 ± 5.0 µm. They are significantly smaller than large cells ($p = 0.000$) but bigger than small dense cells ($p = 0.000$).

220 (6.3%) dead tumoral cells stained within 6 minutes after surgery, present a strong red fluorescent nuclei, but they contain numerous living bacteria. Their size is the biggest among all living or dead cells, with a diameter ($\tilde{x} \pm 1SD$) 18.0 ± 6.8 µm ($p = 0.000$). Each cell contains 75.0 ± 37.9 viable bacteria. Inside their red fluorescent nuclei there are holes, some of them have living bacteria or new nuclei, but most of the big bacterial mass is located in the cytoplasm with strongly green fluorescence around the nuclei.

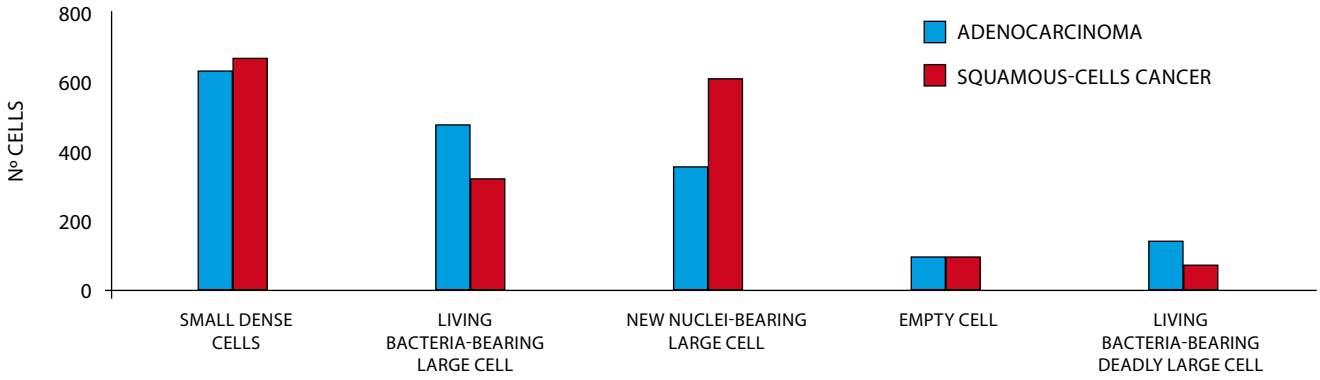
A parallel study was designed for observing living new nuclei inside non-vitality malignant and benign cells. There was viable new nuclei on 278/699 (39.8%) tumors and 0/352 on dead benign cells.

Cell categories and sizes were analyzed according to tumor types: squamous-cell tumors and adenocarcinomas. 1576/3258 alive adenocarcinoma cells are statistically significant, and larger than those from squamous-cell carcinoma: ($\tilde{x} \pm 1SD$) 12.2 ± 6.9 versus 10.4 ± 6.3 µm respectively ($p = 0.000$). Larger adenocarcinoma sizes occur in different cell types except in new nuclei-bearing cells that are similar in both tumors (Table 8/3).

The distribution of different cell morphologies was also different between adenocarcinoma and squamous-cell types. The latter have higher numbers of new nuclei-bearing cells while adenocarcinoma have more cells with bacteria-bearing living and dead cells ($\chi^2 = 121.1$, $p = 0.000$); there are not differences on the percentage of small dense and empty cells (Figure 8/1).

The primary lung cancer sizes in all tumors are statistically correlated with the number of bacteria-living bearing cells and with new nuclei load ($p < 0.05$), and these correlations were also maintained in squamous-cell carcinoma (Tables 8/4 and 8/5). Neither cell type nor any other characteristic was related with primary tumor size or staging (TNM post-surgery).

Figure 8/1. DIFFERENCES OF CELL DISTRIBUTION BETWEEN ADENOCARCINOMAS AND SQUAMOUS-CELLS CANCERS ($\chi^2 = 121.1$, $p = 0.000$)



Gram staining showed Gram-positive cocci inside cells, including new nuclei and small dense cells, although the latter were more difficult to observe, thus careful staining was necessary.

Living bacteria, detected by fluorescent staining, are present in all tumors. Viable bacteria were found inside of cells from 4/20 pieces from neighboring healthy lung. The sensitivity and specificity were 100 and 80% respectively (Table 8/6).

4. Discussion

Syto 9 plus Propidium iodide label human cells and endosymbionts viability. Compared with the uniformity from non-tumoral cells appearance (Figures 8/2 to 8/6), cancer cells show a widespread variation of size, morphology and fluorescent density that is related to different metabolic stages due to living bacteria inside neoplasic cells. As a matter of fact, intracellular bacteria can be found only in a few cases, most of them are extracellular except in four pieces of normal lung, with few bacteria inside benign cells, and some of them were probably macrophages. Living bacteria, are recognized by their green fluorescent, and they are present in all cases studied of lung cancer, thus it is demonstrated that numerous viable bacteria live inside tumoral cells or that cancer cells contain living endosymbionts. In fact, near to 40% of neoplasic cells are small, strongly green fluorescent cells and in most cases no bacteria can be observed. Despite of that, it can prove that these cells have cocci stain with Gram dyes.

Nearly 25% of cells present living endosymbionts inside nuclei and cytoplasm. The number of bacteria are difficult to count, due to the fact that they are aggregates like clusters, that are in part responsible for the large size of these cells, double that of small dense cells. Around 30% of cells show about 3 new nuclei with a mean diameter of 2.2 micrometres and in many cases with close bacterial clusters. Gram stain confirms bacterial clusters, but the most important point is that new nuclei also contain Gram-positive cocci. Another 6.3% are cells stained within a few minutes after tumor resection and are dead with red fluorescent nuclei due to cell membrane damage. It is very common to observe cavities inside of nuclei, and some of them with living bacteria. But, the most outstanding finding is the great amount of viable bacteria around the dead nuclei that increases its size above all malignant cells. It means that living bacteria can kill tumoral cells and it also confirms active endonucleosymbionts. Around 5% of tumor viable cells with a weak green fluorescence represent empty cells after the release of bacterial clusters and new nuclei and consequently their size is intermediate between large and small cells.

In short, in comparison with the uniformity of non-tumoral cells, there is a wide variation of morphologies, sizes and viability of cancer cells that express a complex cellular cycle due to the relationship between tumor and its endonucleosymbionts.

This cycle can be summarized in five steps:

1. Small dense cells.

They comprise a constant proportion of about 40% of tumoral mass of the most frequent cancers: squamous-cell and adenocarcinoma. These cells are small (mean: 7 µm) with homogeneous dense fluorescence (Figures 8/17 and 8/18), but in some cases living bacteria can be observed at its edge (Figures 8/19 to 8/21). Gram stains Gram-positive cocci inside the cell (Figure 8/22 to 8/42). Theese cells grow and develop large cells characteristics.

2. Bacterial clusters-bearing living cells.

They appear in near to 20% of all cells, and they are more frequent among adenocarcinomas. Their green fluorescence leads to observable groups of viable bacteria, around 70 per cell. Most of isolated or crowded microbes are in the cytoplasm and they contribute to increasing cell diameters up to double those of small dense cells. The swollen cytoplasm are brittle (see Chapter 5), in consequence dozen of germs can be released into extra-cellular space and they can reach faraway places, due to their small size, approximately 1 µm. Thus, they are the most important candidates to produce large distance metastases, through the bloodstream or the lymph system. This item must be verified, because our selective surgical cases ruled out widespread cancers. Bacteria-bearing living cells correlate inversely with primary tumor size in all studied tumors, and especially in squamous-cell carcinomas. This fact supports that increased in this type of cells (related more with widespread cancer) directly involves a decrease in the number of new nuclei-bearing cells, because both are different stages from the same cell, and are also positively correlated with local growth (see below). Figures 8/43 to 8/58.

Gram staining shows crowded Gram-positive cocci inside cells. It is noteworthy that extra-nuclei cocci are larger than those inside of nuclei (see Chapter 6), and they are the infective forms, like Holospore cycle (see below). It is necessary to insist that microbes-bearing cells and new nuclei-bearing cells are the same cells, and one or the other morphology are only sequences of the same process. Figures 8/59 to 8/69 are samples of intranuclear cocci, while Figures 8/70 to 8/87 are larger cocci placed out of nuclei.

3. New nuclei-bearing cells.

Around 30% of cancer cells contain about 3 new nuclei per cell, with a diameter near to 2.5 micrometers each new nucleus. Some of them look like nucleoles, but they are a consequence of nuclear division, and they will be eventually released and turned into small dense cells. The main characteristics observed are:

A. More than one third of dead tumoral cells keep a red fluorescent nucleus and green fluorescent new nuclei inside them. This phenomenon has never been observed among dead benign cells (Figures 8/220 to 8/223).

B. The numbers of new nuclei have a mean of 2.4 per cell, but they can reach near to thirty.

C. The size varies from 1 to more than 10 µm (Figures 8/88 to 8/94).

D. Most of them are round, and when they are numerous and close to each other the cells are plenty of "balloon release" structures (Figures 8/95 to 8/98); but they acquired several morphologies: rods (Figures 8/99 to 8/104); X-like morphology (Figures 8/105 and 8/106); Y-like forms (Figures 8/107 and 8/108); irregular morphology (Figures 8/109 to 8/111).

E. New nuclei formation is the consequence of old nuclei narrowing until their complete division, and this can be present as:

- Bipolar division. It is the less commonly observed and a nucleus generates two new ones (Figure 8/112 to 8/117).
- Long rods with several narrowing sections that progressively form new nuclei (Figures 8/118 to 8/129).
- Multiple close rods, with complex forms like X or Y, suffer divisions and originate several nuclei (Figures 8/130 to 8/140).
- Multipolar division, where all cells have plenty of rounded nuclei giving the "balloon release" image (Figures 8/141 to 8/148).

These types of nuclear division are frequently observed in tumors with lacto-orcein, but much better with Gram staining (Figures 8/149 to 8/186).

The new nuclei leave the cells, at least in two ways:

Firstly, by disappearance of the cell membrane and subsequent young nuclei release. This occur in poor viability cells (Figures 8/187and 8/188).

Secondly, when nuclei go out through membrane "holes", apparently without reduction of cell vitality (Figures 8/189 and 8/190). This process can be observed better with Gram and lacto-orcein dyes, where one or several young nuclei go out with part of the cytoplasm cell, rather than trough the "holes" seen with fluorescent stains (Figures 8/191 to 8/202).

Gram staining also allows observing bipolar or multipolar divisions, where old nucleus split into parts and Gram-positive cocci are distributed among the new nuclei. Obviously, this division is quite effective, with equal distribution of bacteria in the new nuclei, as it occurs in reproductive forms of Holospora sp.[3]

The differences between microbe clusters and new nuclei ones, are that latter are inside new nuclei and these bacteria are reproductive forms, where endonucleosymbions cocci produce new neoplasic cells. Extranuclei cocci are groups of cocci and are infective forms, containing larger cocci, probably due to stop in binary bacterial division, as it occurs in Holosporas cycle (see Chapters 11 and 12).

As it can be expected, new nuclei-bearing cells have a statistical significance on the size of all primary lung cancer cases studied, and this relationship is also true in squamous-cell cancer. This fact is explained because the large cells constitute near to 1/4 of the tumoral mass, with diameter about 15 µm and limited mobility and thus responsible of increased number of new cells with endosymbionts and local growth of cancers. The new nuclei load (new nuclei percentage by mean of nuclei per cell) has a mean value of around 70/100 tumoral cells. But this figure can increase up to five times more. As a matter of fact, some cases of new nuclei-bearing cells can rise up to 75% of all cells and any tumoral cells carry two or three dozen of new nuclei, with full capacity to grow and spread further. Gram-positive endosymbionts generate more and more parasitic nuclei, because cocci affect nuclear morphology and functions. Cocci remain inside the cells and their descendants persistently "reproduce" into new endonucleosymbionts. Due to their active binary division these reproductive forms are small cocci and are crowded inside the nuclei. Reproductive forms, under special circumstances can originate infective forms, where cocci are three times larger, and have potential to go outside the cells and "infect" new healthy cells.

Theoretically, one single cell can produce about one hundred living infective forms and near to thirty new cells with reproductive endosymbionts. This scalating progression can explained the inexorable natural history of malignant tumors. It must be noted that therapies directed only to impairing malignant cells, massively release living extranuclear cocci and new nuclei with plenty of endonucleosymbiont cocci, and thus favor its wider spread. This is in line with the mild impact that some chemotherapies and other similar treatments have on the survival rate and quality of life in most types of tumors.

4. Empty viable cells.

They are 5% of all cells, and despite of their viability, they are the result of bacteria and new nuclei release. Gram dye shows big vacuoles, practically without bacteria, and they represent the final stage of the tumoral cell cycle (Figures 8/203 to 8/207).

5. Viable bacteria-bearing large dead cells.

Smear and staining done on samples obtained only a few minutes after surgery, show near to 5% of neoplasic cells with dense red fluorescent nuclei containing, high numbers of living bacteria, gathered around the dead nuclei; some isolated bacteria are seen inside holes that are frequently found in theese nuclei (Figures 8/208 to 8/216). An outstanding fact is that these cells are the biggest of all other benign or malignant cells, especially in adenocarcinomas, where their diameters are nearly 20 µm, in part due to their massive bacterial load. A similar situation occurs when endosymbionts-bearers *Paramecium aurelia* are placed in a poor medium. In that case the paramecia may die, but before death it is found that endosymbionts have increased to exceedingly high numbers, and are probably the cause of cell death (see Chapter 11). Figures 8/217 to 8/219.

[3] Gortz Hd. *Endonuclear symbionts in ciliates.* Int Rev Cytol 1983; (suppl 14): 145-76.

The big bacterial load released by the disappearance of the damage cell membrane (Figures 8/224 to 8/226). The natural or induced cancer cell necrobiosis probably doesn't mean a better prognosis for patients, but an opportunity for new tumor spread further.

Heterogeneous sizes, morphologies and viability of tumor cells are expression of a complex cycle of cocci-cancer cell interaction.

Living bacteria as main inducers of this cycle can be observed in tumors using fluorescent dye (Syto plus Propidium iodide) with a sensitivity, specificity and odds ratio of 100%, 80% and undefined respectively (Table 8/6).

Table 8/1. GENERAL CHARACTERISTICS OF 20 CASES. SYTO 9 PLUS PROPIDIUM IODIDE STAINING.

Nº	AGE (years)	GENDER	TYPE OF TUMOR	STAGING			PRIMARY TUMOR		STAINING TIME AFTER SURGERY (min)	TYPE OF LUNG RESECTION
				T	N	M	Φ MAXIMAL (cm)	AREA (cm^2)		
49	70	MALE	ADENOCARCINOMA	2	0	X	3	9	5760	NEUMONECTOMY
50	63	MALE	SQUAMOUS CELL	2	0	X	3	9	5760	LOBECTOMY
51	55	MALE	ADENOCARCINOMA	1	0	X	4.5	15.8	240	LOBECTOMY
52	76	MALE	ADENOCARCINOMA	2	0	X	4	16	240	LOBECTOMY
53	78	MALE	ADENOCARCINOMA	2	0	X	3.5	12.3	30	ATYPICAL RESECTION
54	81	MALE	SQUAMOUS CELL	2	0	X	5.4	24.3	40	ATYPICAL RESECTION
55	58	MALE	ADENOCARCINOMA	1	0	X	2.5	5.5	6	LOBECTOMY
56	71	MALE	SQUAMOUS CELL	2	0	X	2	4	7	NEUMONECTOMY
57	52	MALE	ADENOCARCINOMA	2	0	X	4.2	10.5	3	LOBECTOMY
58	70	MALE	SQUAMOUS CELL	2	0	X	3.5	10.5	31	ATYPICAL RESECTION
59	61	MALE	SQUAMOUS CELL	3	0	X	5	15	45	LOBECTOMY
60	78	MALE	SQUAMOUS CELL	2	1	X	5.5	22	40	LOBECTOMY
61	62	FEMALE	ADENOCARCINOMA	2	0	X	6	24	40	NEUMONECTOMY
62	54	MALE	SQUAMOUS CELL	1	0	X	2.5	5	32	NEUMONECTOMY
63	51	MALE	ADENOCARCINOMA	3	0	X	3.5	10.2	1440	NEUMONECTOMY
64	74	MALE	ADENOCARCINOMA	2	1	X	4	14.8	1440	LOBECTOMY
65	65	MALE	ADENOCARCINOMA	3	1	X	4	14	19	LOBECTOMY
66	52	FEMALE	ADENOCARCINOMA	1	2	X	2.5	5	1080	LOBECTOMY
67	77	MALE	SQUAMOUS CELL	2	0	X	4.5	15.8	36	ATYPICAL RESECTION
68	57	FEMALE	ADENOCARCINOMA	2	2	X	3.1	7.75	41	ATYPICAL RESECTION

Table 8/2. SOCIODEMOGRAPHICS AND TUMOR CHARACTERISTICS. SYTO 9 PLUS PROPIDIUM IODIDE STAINING.

AGE (years)	MEAN ± 1SD. (MAXIMUM, MINIMUM)	65.5±10.0 (51-81)		
GENDER	FEMALE	3 (15.0%)		
	MALE	17 (85.0%)		
DATE OF SURGERY	2010	13 (65.0%)		
	2011	7 (35.0%)		
TYPE OF TUMOR	ADENOCARCINOMA	12 (60.0%)		
	SQUAMOUS – CELL CANCER	8 (40.0%)		
TNM DESCRIPTOR		T	N	M
	0		15 (75.0%)	
	1	4 (20.0%)	3 (15.0%)	
	2	13 (65.0%)	2 (10.0%)	
	3	3 (15.0%)		
	4			
	X			20 (100%)
TYPE OF LUNG RESECTION	ATYPICAL RESECTION	3 (15.0%)		
	LOBECTOMY	12 (60.0%)		
	NEUMONECTOMY	5 (25.0%)		

Table 8/3. CANCER AND NON-TUMORAL CELL TYPE. SYTO 9 PLUS PROPIDIUM IODIDE STAINING.

TUMOR TYPE	**CELL VITALITY**	**Nº CELLS** (%) **DIAMETER** $\tilde{X}$ ± 1SD µm			**STATISTICAL SIGNIFICANCE**
		TOTAL TUMORS	ADENOCARCINOMA	SQUAMOUS-CELL	
SMALL DENSE CELLS	ALIVE	1313 (37.5%) 6.9 ± 4.0	640 (37.1%) 7.3 ± 4.8	673 (37.8%) 6.6 ± 3.0	P = 0.002
LIVING BACTERIA-BEARING LARGE CELL	ALIVE	803 (22.9%) 14.9 ± 7.5	479 (27.8%) 17.4 ± 6.8	324 (18.2%) 11.3 ± 6.8	P = 0.000
NEW NUCLEI-BEARING LARGE CELL	ALIVE	972 (27.7%) 14.3 ± 5.7	359 (20.8%) 14.5 ± 4.4	613 (34.4%) 14.2 ± 6.4	P = 0.473
EMPTY CELL	ALIVE	197 (5.6%) 10.5 ± 5.0	98 (5.7%) 11.2 ± 4.5	99 (5.6%) 9.8 ± 5.3	P = 0.048
LIVING BACTERIA-BEARING DEADLY LARGE CELL	DEATH	220 (6.3%) 18.0 ± 6.8	147 (8.5%) 20.5 ± 6.3	73 (4.1%) 12.9 ± 4.5	P = 0.000
NON TUMORAL CELLS	ALIVE	563 10.6 ± 4.6			

Table 8/4. PRIMARY TUMOR SIZE (MAXIMAL DIAMETER OR AREA) AND PERCENTAGE OF ALIVE BACTERIA-BEARING LARGE CELLS AND NEW NUCLEI LOAD IN TOTAL TUMORS (ADENOCARCINOMAS, SQUAMOUS-CELL CANCERS). SYTO 9 PLUS PROPIDIUM IODIDE STAINING.

	ADENOCARCINOMA $\tilde{X} \pm 1SD$	SQUAMOUS-CELL CANCER $\tilde{X} \pm 1SD$	TOTAL TUMOR $\tilde{X} \pm 1SD$
AGE (yr)	62.4 ± 9.8	69.4 ± 9.4	65.2 ± 10.0
MAXIMAL DIAMETER (cm) PRIMARY TUMOR	3.7 ± 1.0	3.9 ± 1.4	3.8 ± 1.1
AREA (cm^2) PRIMARY TUMOR	12.1 ± 5.3	13.2 ± 7.4	12.5 ± 6.1
NEW NUCLEI /CELL	2.3 ± 0.7	2.6 ± 1.3	2.4 ± 0.9
NEW NUCLEI LOAD / 100 TUMORAL CELL	72.2 ± 40.7	98.2 ± 90.3	83.4 ± 64.9
% LIVING BACTERIA-BEARING LARGE CELL	27.8%	18.2%	22.9%

Table 8/5. CORRELATION AMONG PRIMARY TUMOR SIZE (MAXIMAL DIAMETER OR AREA) AND PERCENTAGE OF ALIVE BACTERIA-BEARING LARGE CELLS AND NEW NUCLEI LOAD. SYTO 9 PLUS PROPIDIUM IODIDE STAINING.

CORRELATIONS DATA	ADENOCARCINOMA	SQUAMOUS-CELL CANCER	TOTAL TUMORS
MAXIMAL DIAMETER (cm) PRIMARY TUMOR vs NEW NUCLEI / CELL	NO SIGNIFICANT DIFFERENCES	NO SIGNIFICANT DIFFERENCES	NO SIGNIFICANT DIFFERENCES
AREA (cm^2) PRIMARY TUMOR vs NEW NUCLEI / CELL	NO SIGNIFICANT DIFFERENCES	NO SIGNIFICANT DIFFERENCES	NO SIGNIFICANT DIFFERENCES
MAXIMAL DIAMETER (cm) PRIMARY TUMOR vs NEW NUCLEI LOAD / 100 TUMORAL CELL	NO SIGNIFICANT DIFFERENCES	r = 0.751 P = 0.085	r = 0.575 P = 0.031
AREA (cm^2) PRIMARY TUMOR vs NEW NUCLEI LOAD / 100 TUMORAL CELL	NO SIGNIFICANT DIFFERENCES	r = 0.913 P = 0.011	r = 0.649 P = 0.012
MAXIMAL DIAMETER (cm) PRIMARY TUMOR vs % LIVING BACTERIA-BEARING LARGE CELL	NO SIGNIFICANT DIFFERENCES	r = -0.786 P = 0.021	r = -0.507 P = 0.027
AREA (cm^2) PRIMARY TUMOR vs % LIVING BACTERIA-BEARING LARGE CELL	NO SIGNIFICANT DIFFERENCES	r = -0.746 P = 0.033	r = -0.452 P = 0.052

Table 8/6. LIVING BACTERIA DETECTED BY SYTO 9 PLUS PROPIDIUM IODIDE STAINING.

LIVING BACTERIA	TUMOR	NON-TUMOR LUNG	TOTAL
POSITIVE	20	4	**24**
NEGATIVE	0	16	**16**
TOTAL	20	20	**40**

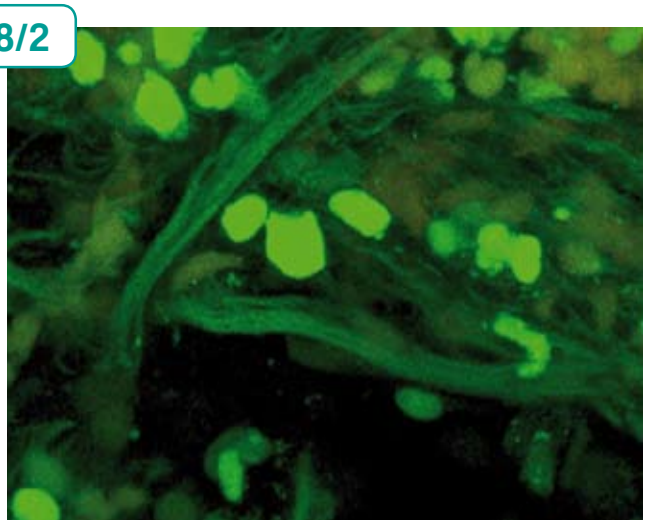
Sensibility: *100.0 % 95% CI (83% to 100%)*
Specificity: *80.0 % 95% CI (56% to 94%)*
Likelihood ratio (+): *5*
Likelihood ratio (-): *0.00*
Likelihood ratio (+) / Likelihood ratio (-): *Undefined*
OR: *Undefined*
χ^2 ***(correction Yates):*** *23 (P = 0.000)*

Syto 9 plus Propidium iodide staining

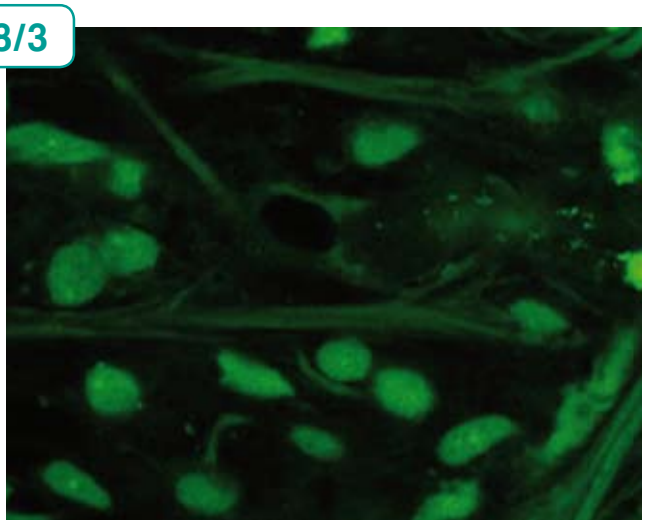
FIGURES 8/2 TO 8/21

→ **Typical aspect of non-tumoral cells.** They appear with uniform density (Figures **8/2** to **8/6**)

8/2



8/3



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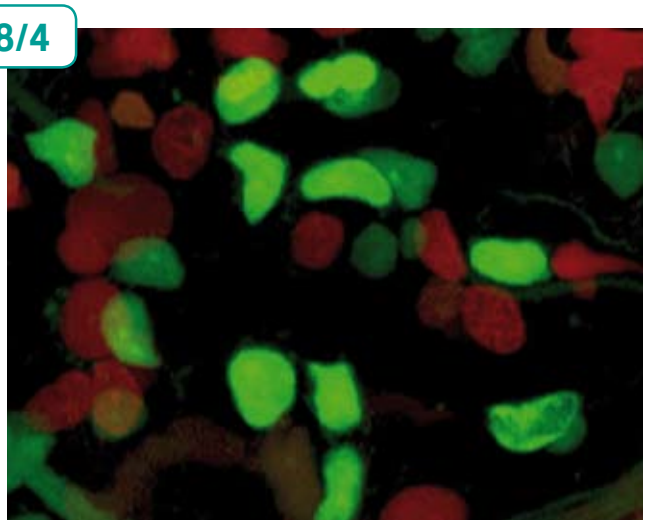

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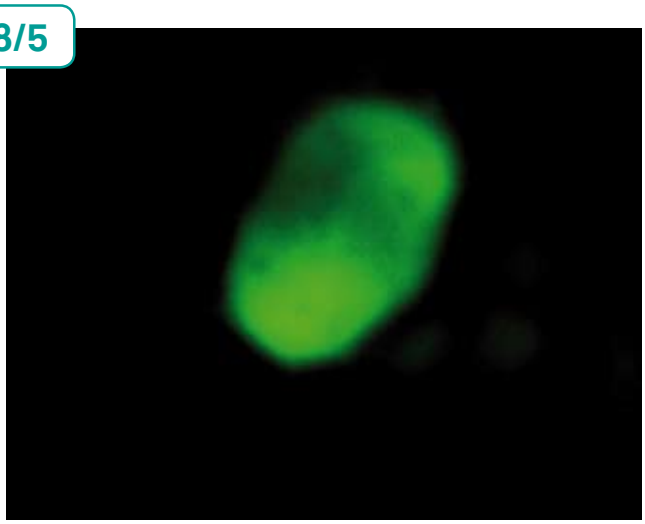

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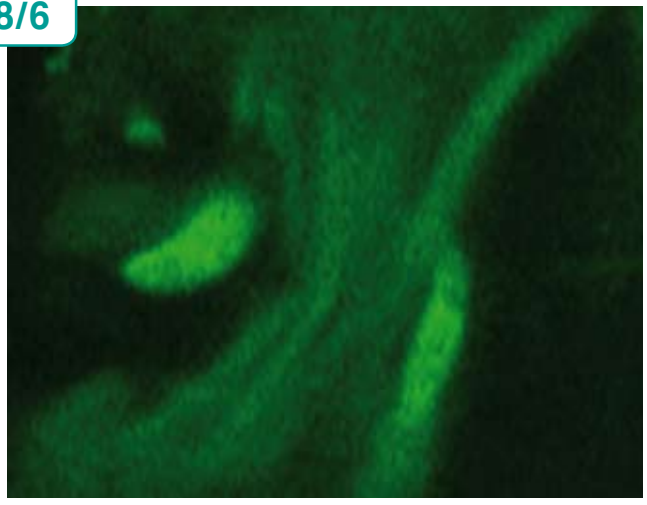
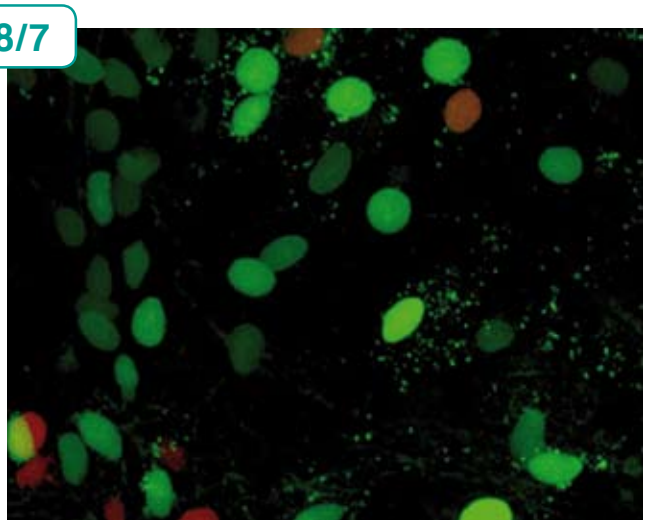
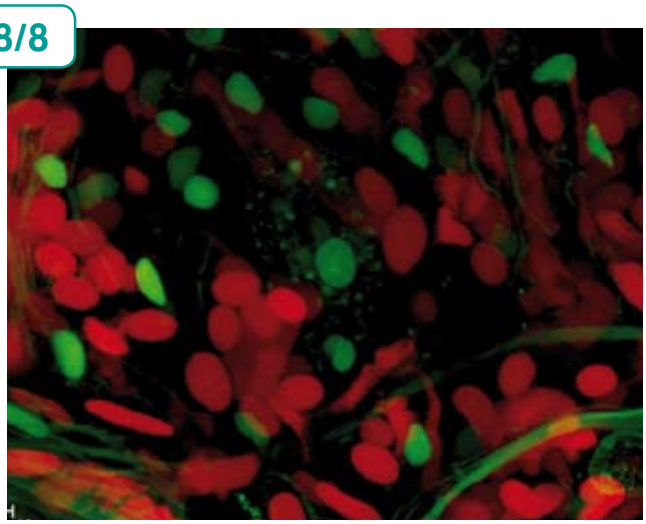


→ **Living bacteria outside benign cells** (Figures **8/7** to **8/10**)

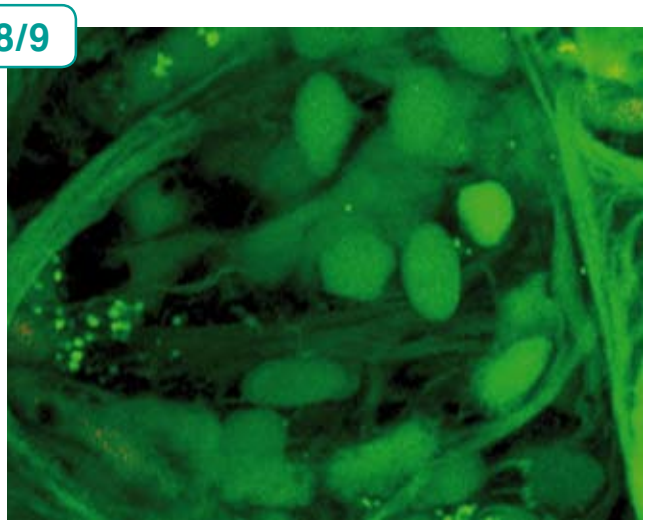
8/7



8/8



8/9



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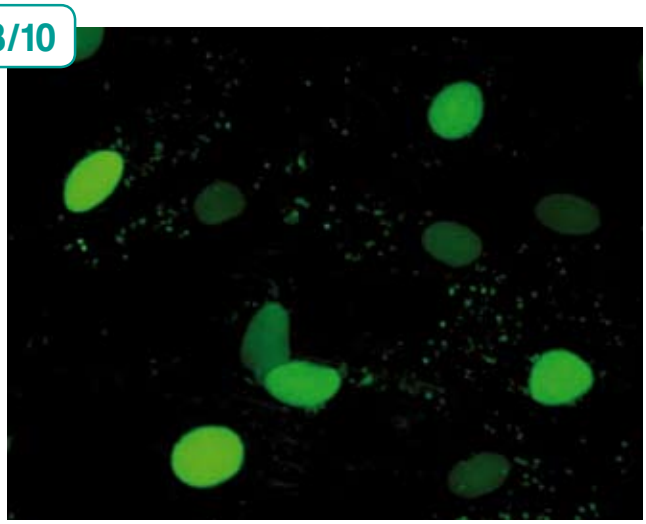
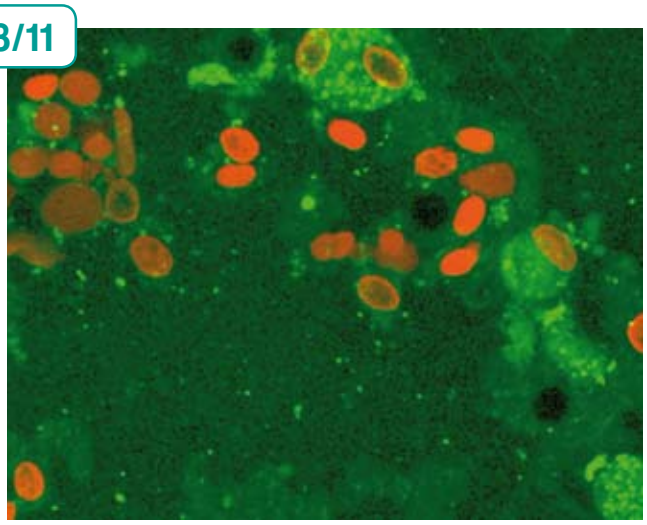


→ **Scarce living bacteria inside non-tumoral cells** (some of them are probably lung macrophages) (Figures **8/11** to **8/16**)

8/11



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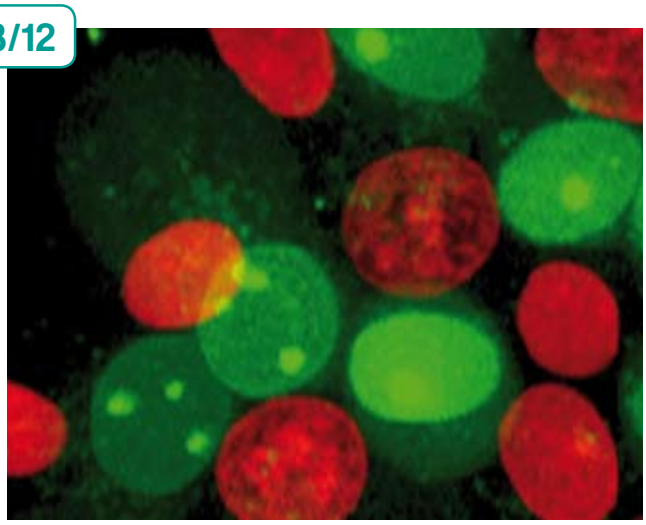

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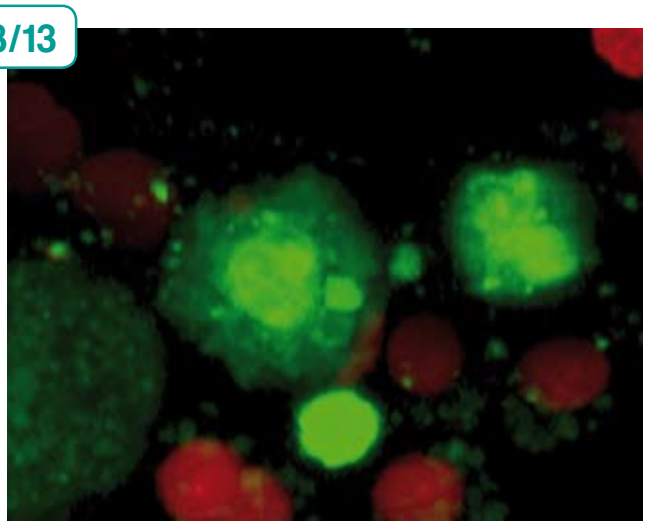

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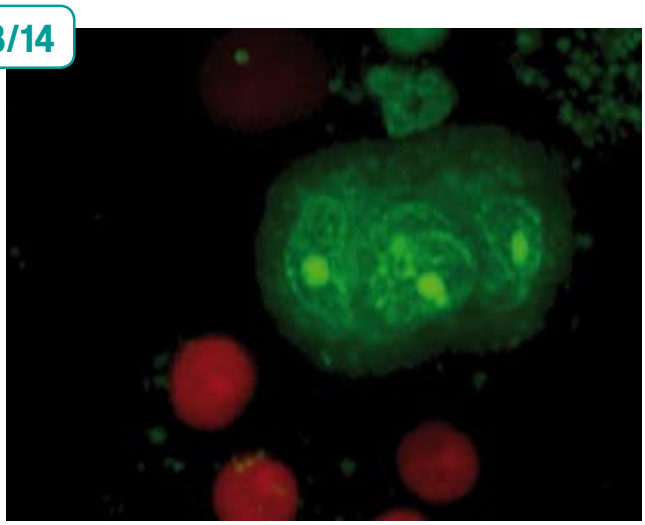

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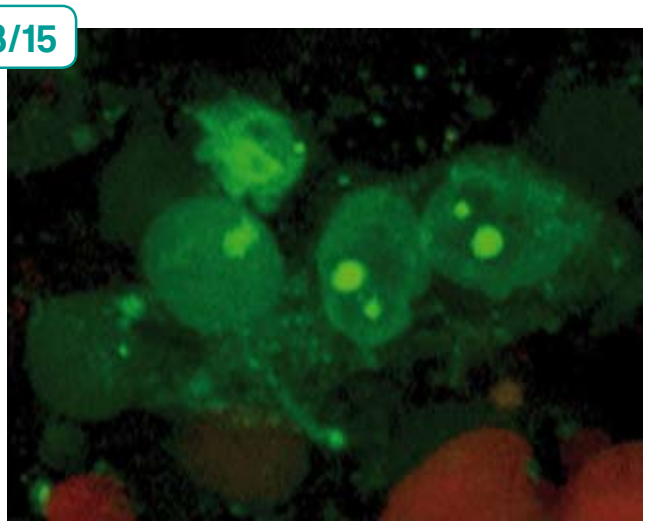

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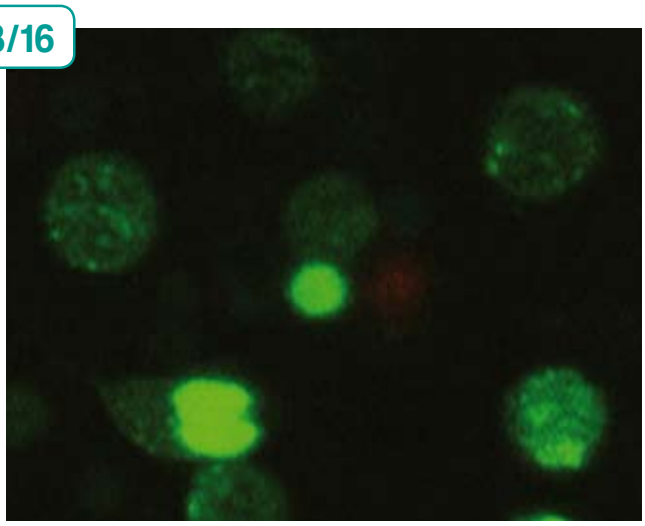
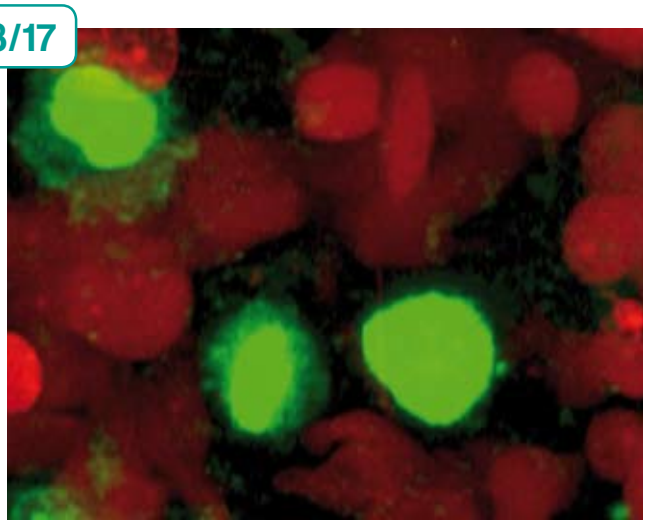


→ **Small dense tumor cells** (Figures **8/17** and **8/18**)

8/17



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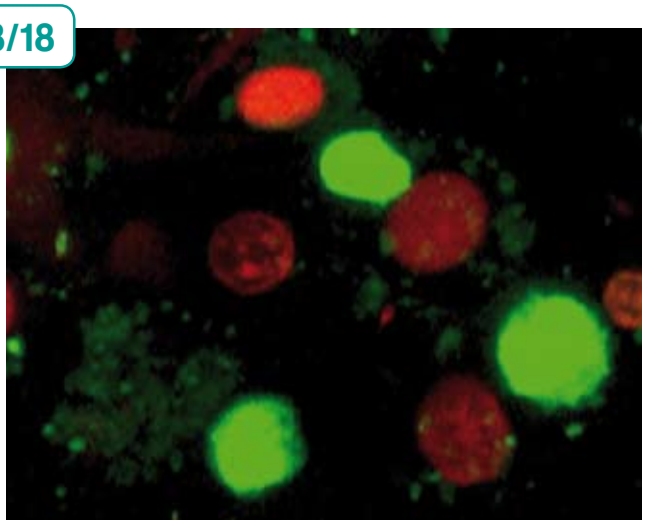
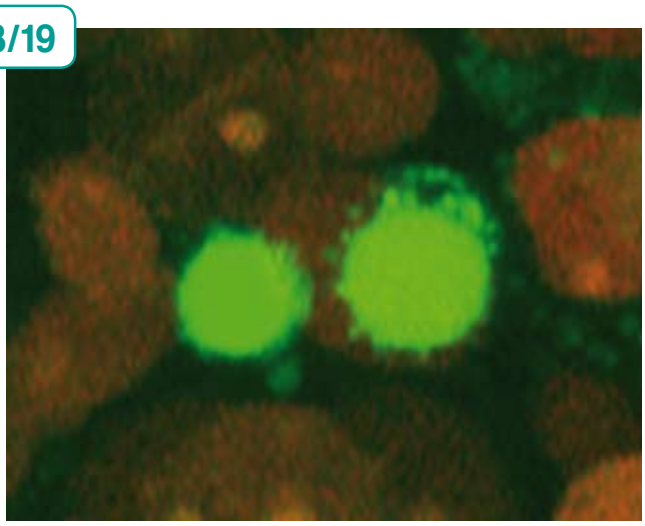


→ **Occasional bacteria seen at the border of small dense tumor cells** (Figures **8/19** to **8/21**)

8/19



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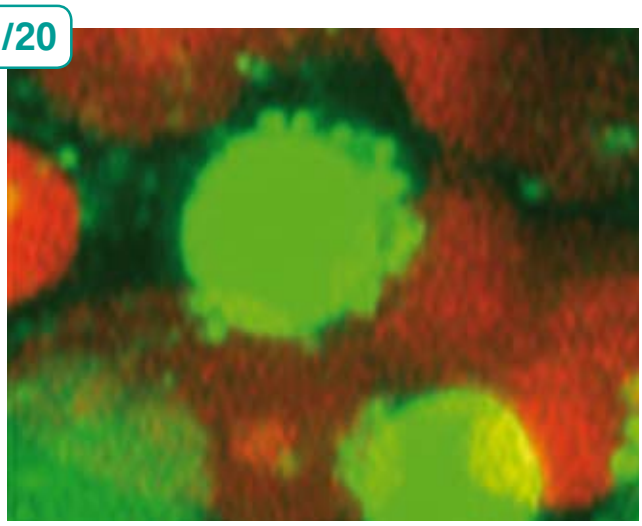
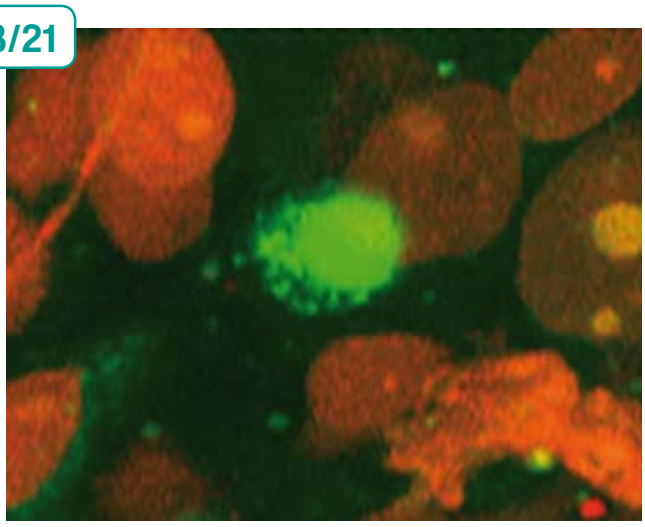


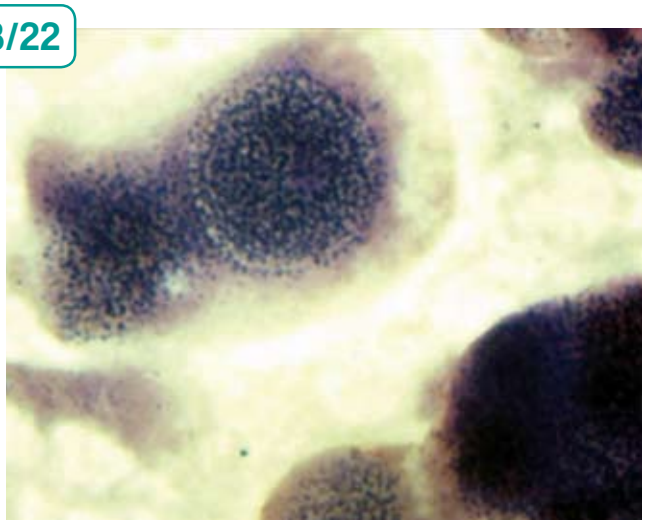
8/21



Small dense tumor cells containing many Gram-positive cocci (×2000 Gram staining) ↓

FIGURES **8/22** TO **8/42**

8/22



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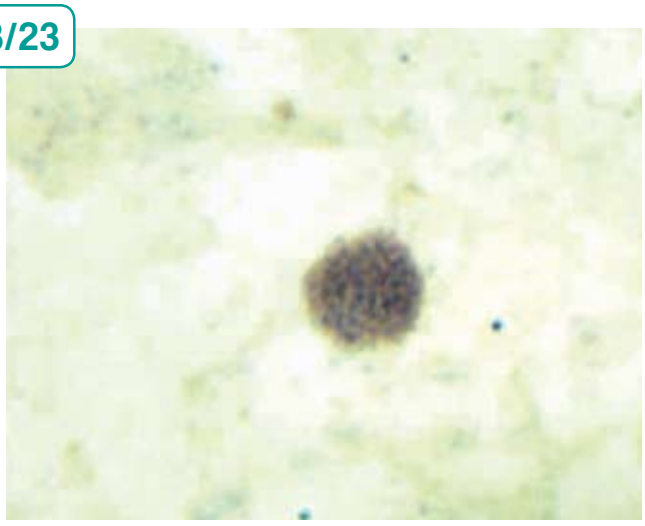
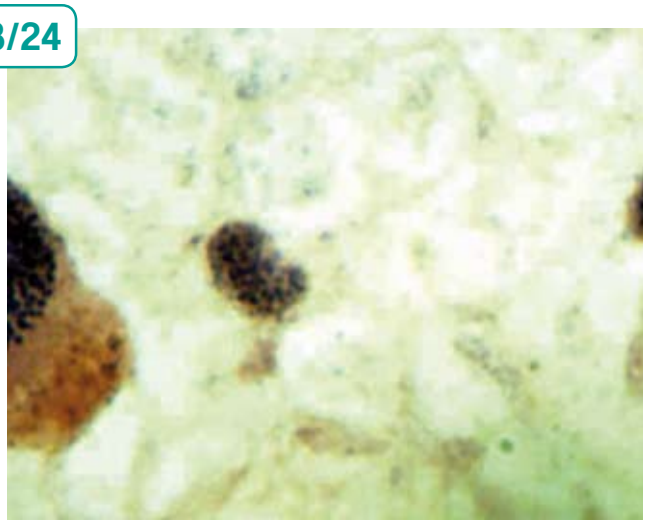
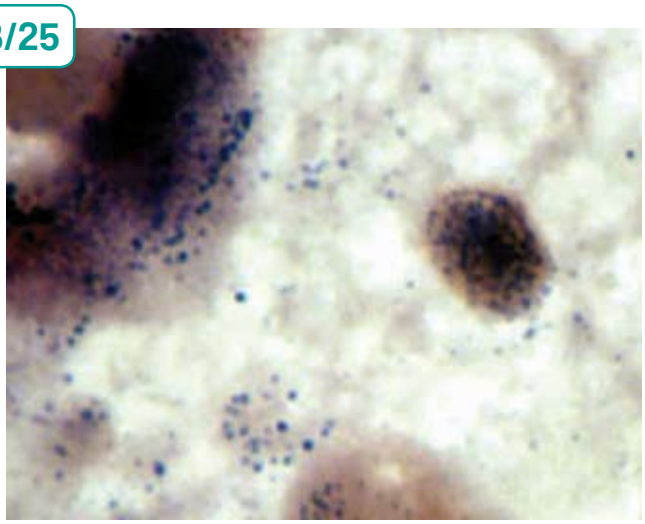


8/24



8/25



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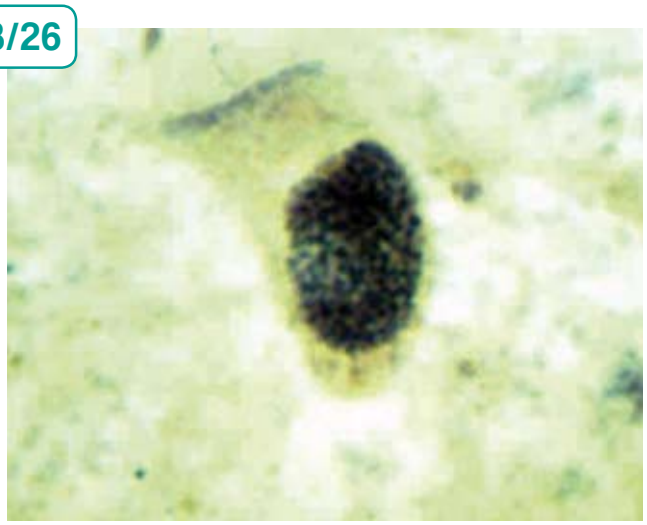

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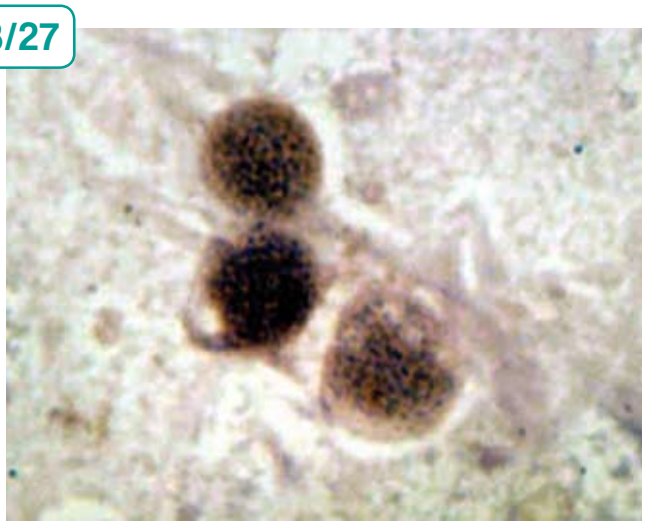

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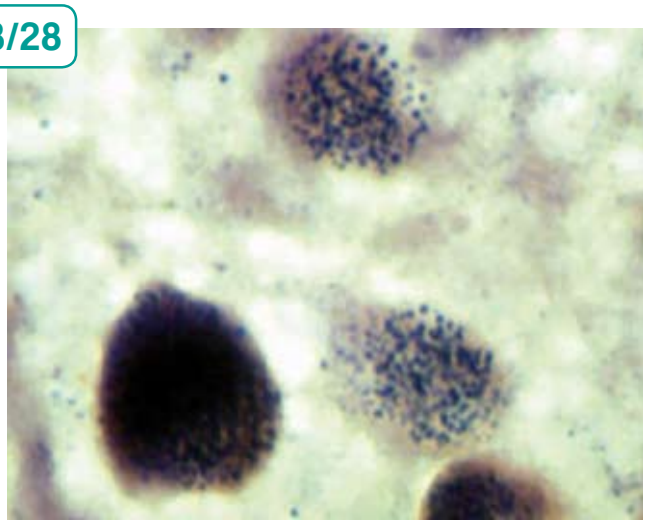

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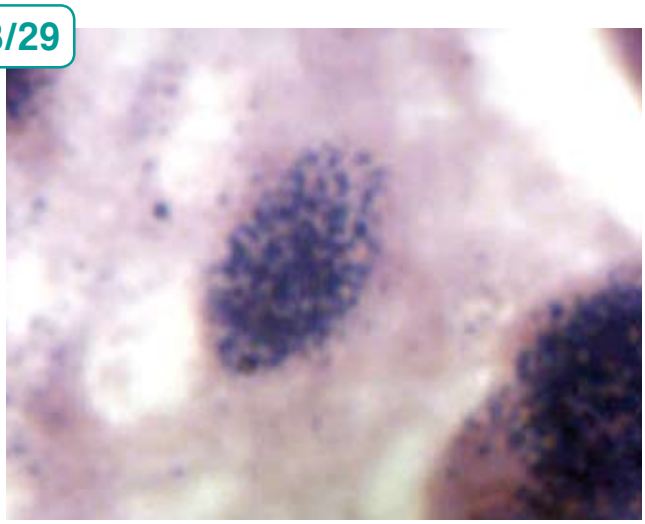

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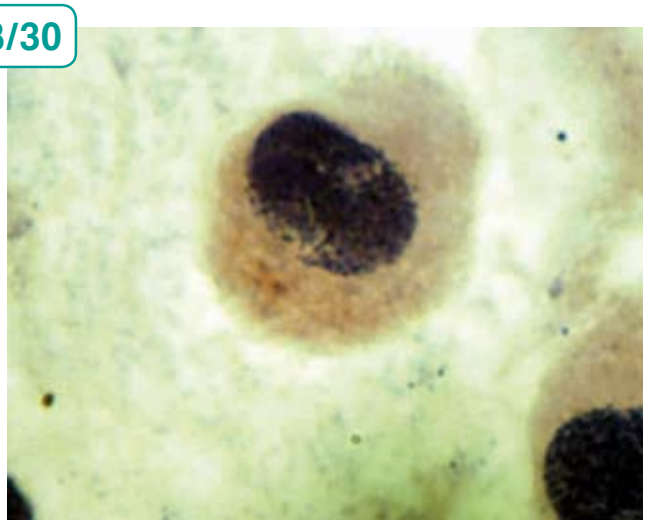

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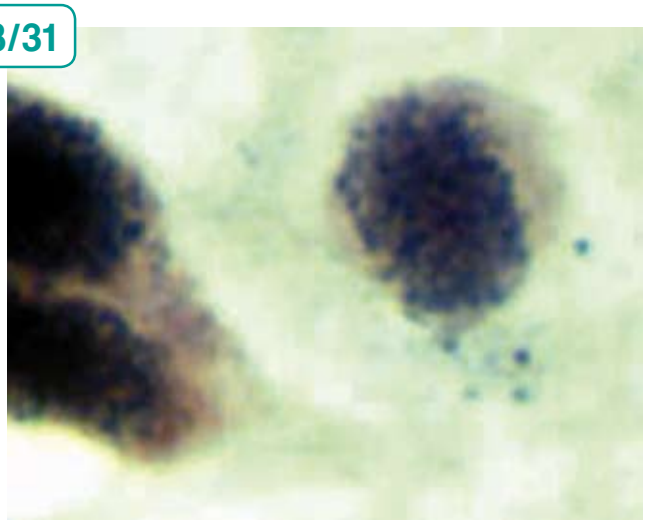

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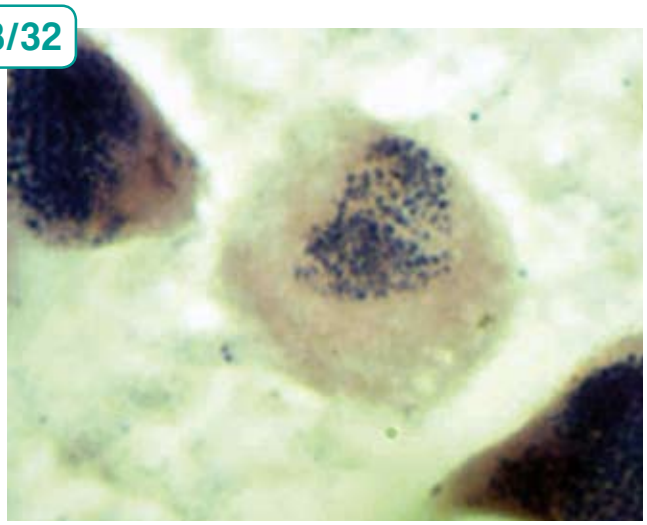
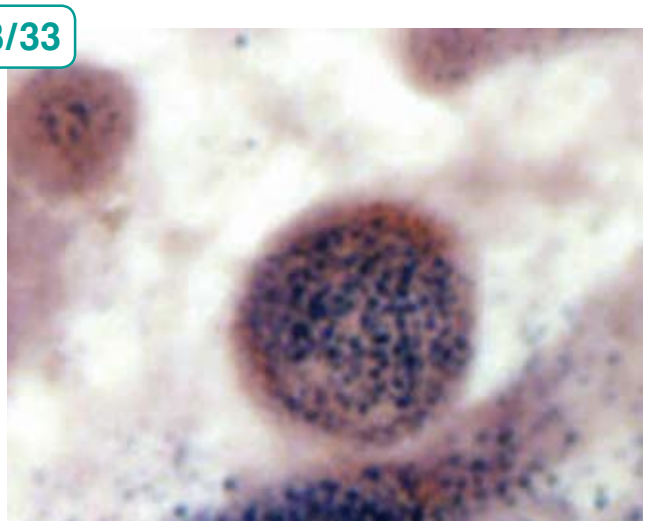


8/33



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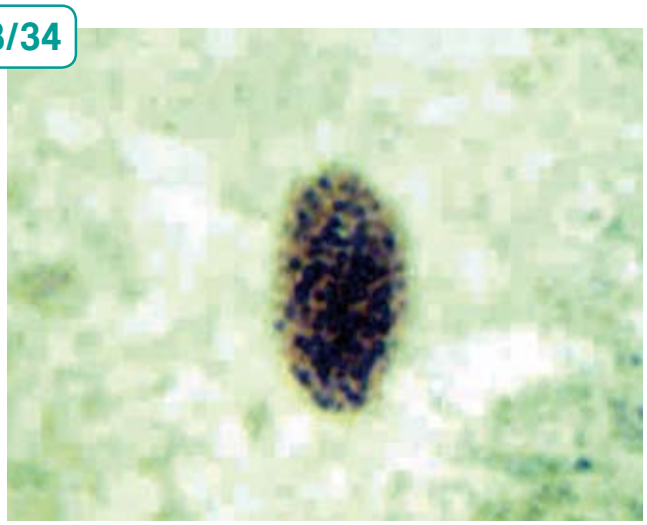

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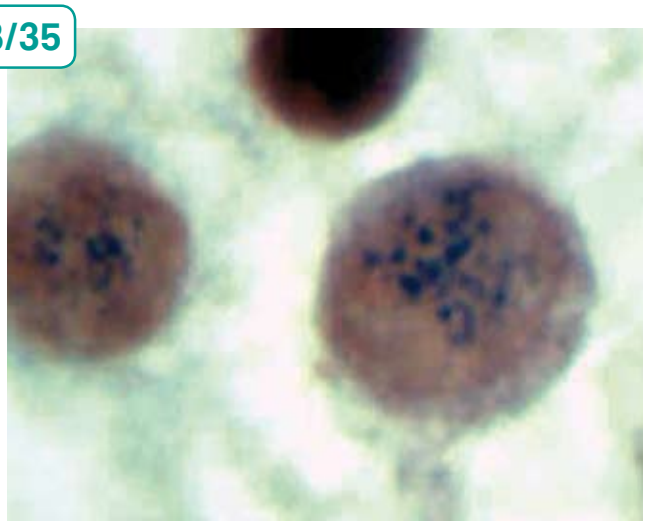

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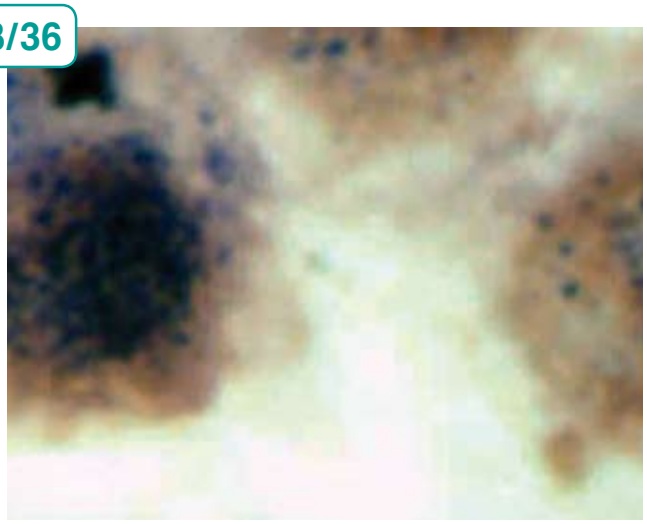

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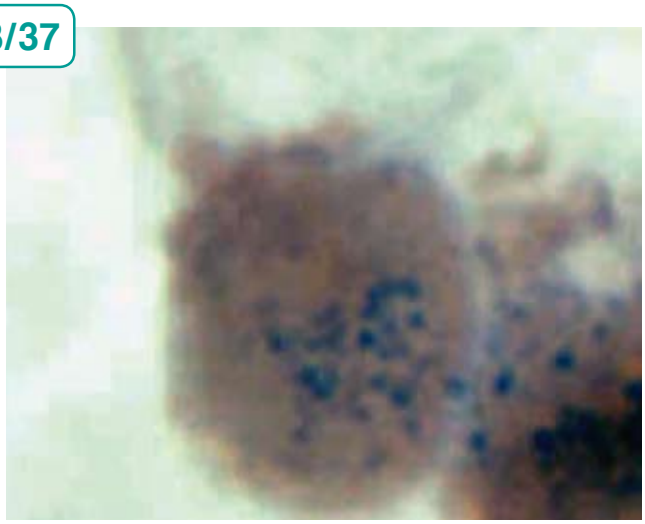

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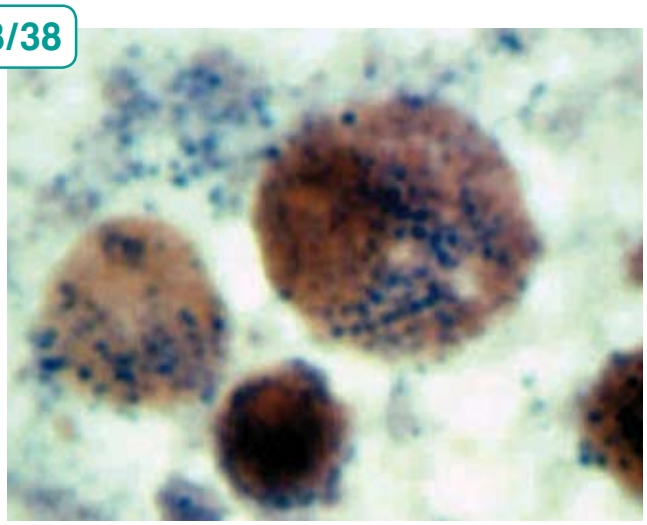

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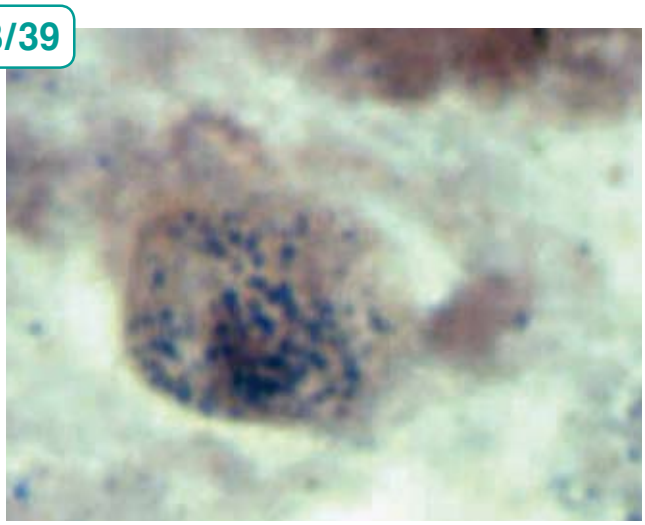
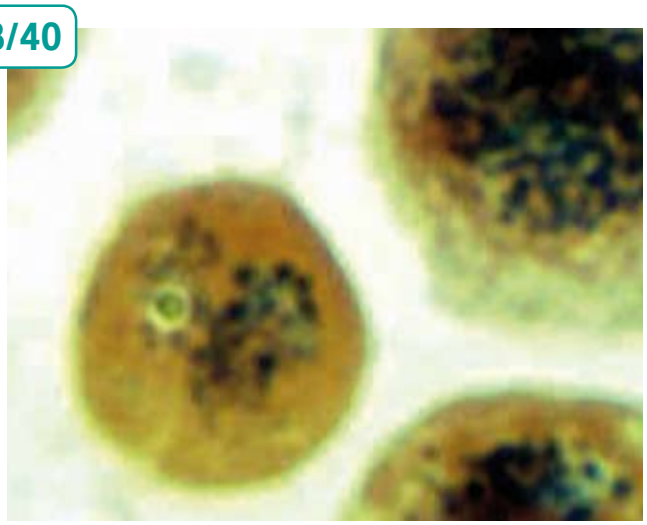


8/40



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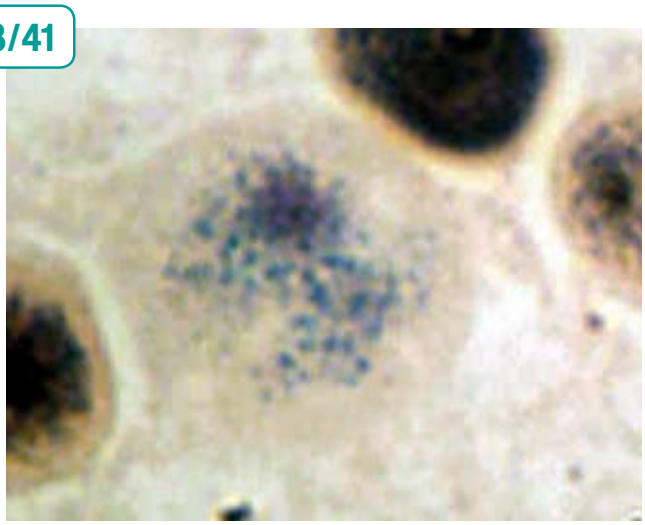

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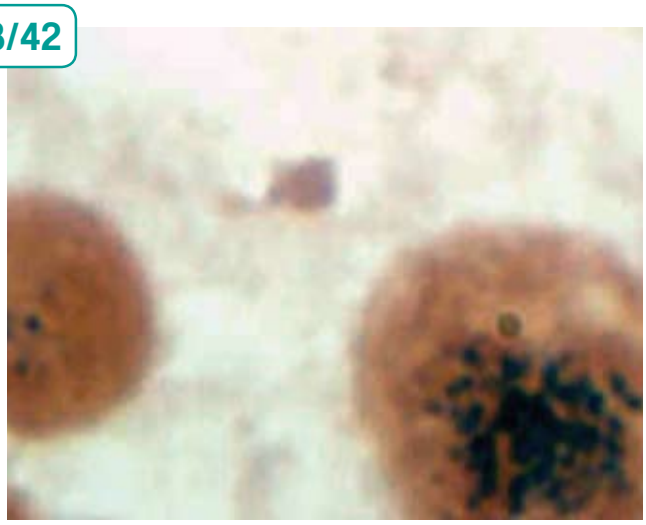
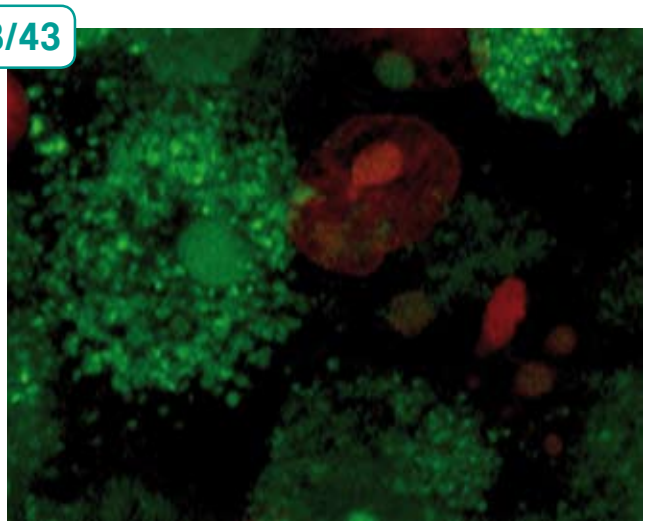


Large living tumor cells-bearing bacteria clusters (Syto 9 plus Propidium iodide)

FIGURES 8/43 TO 8/58

8/43



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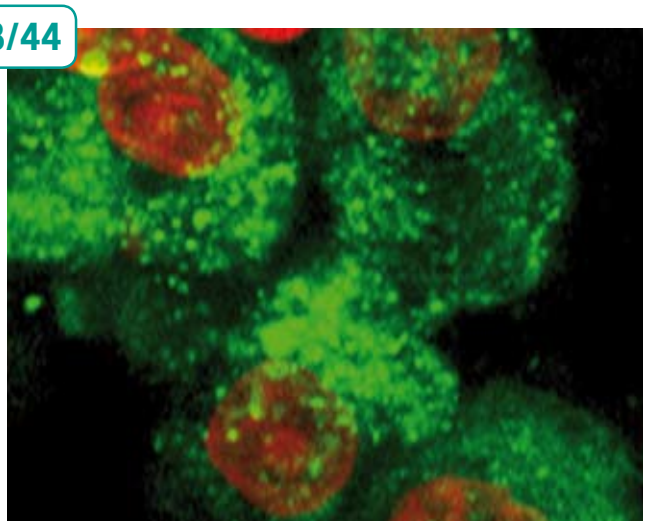

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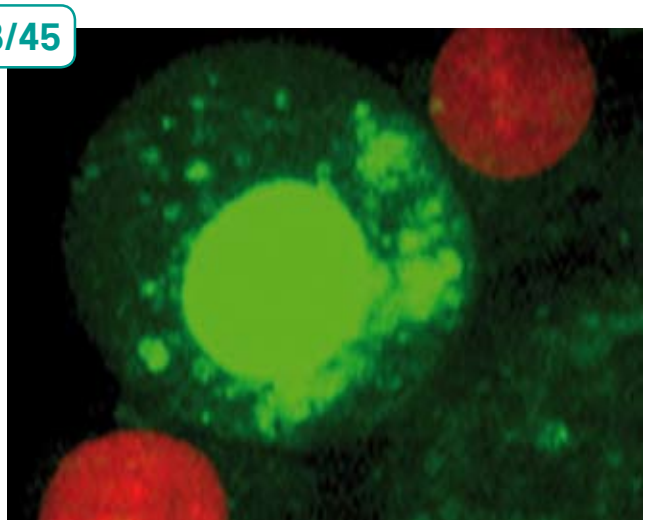

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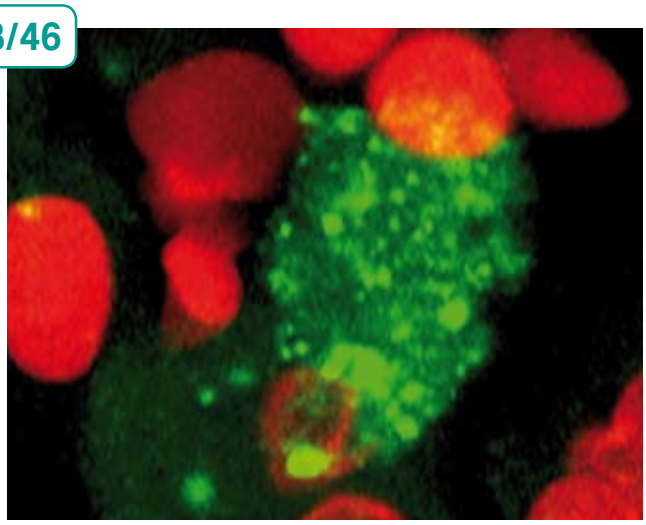

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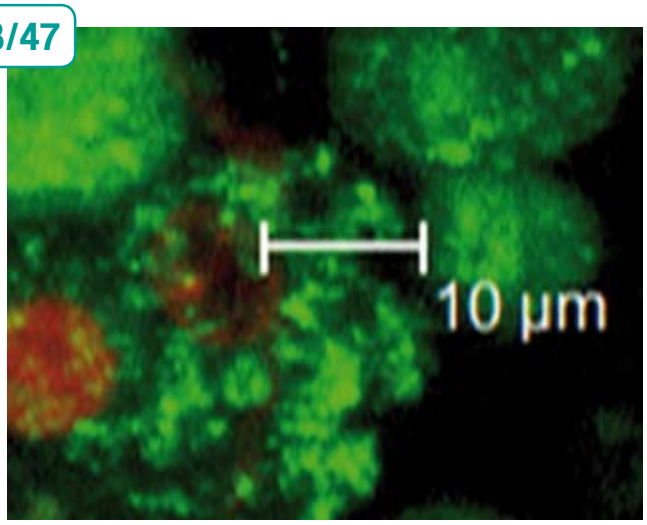

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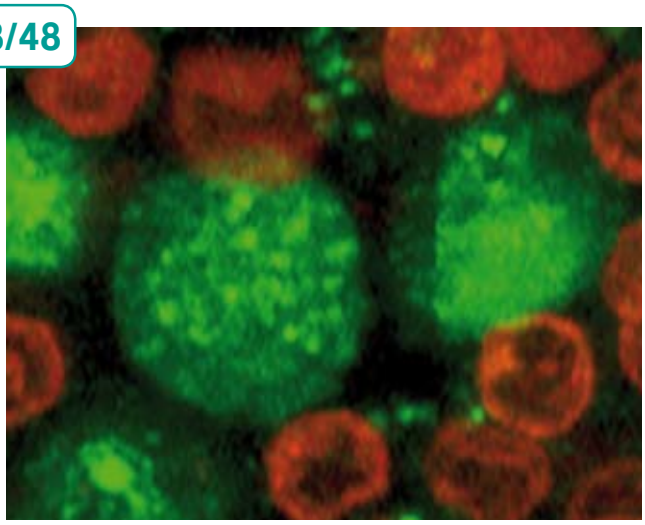

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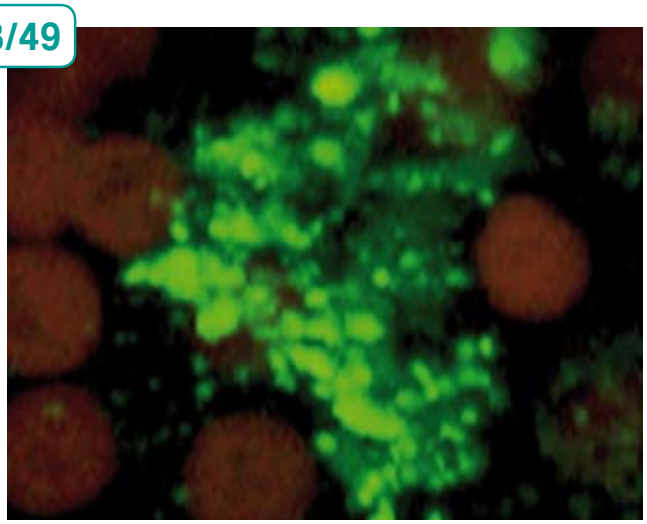

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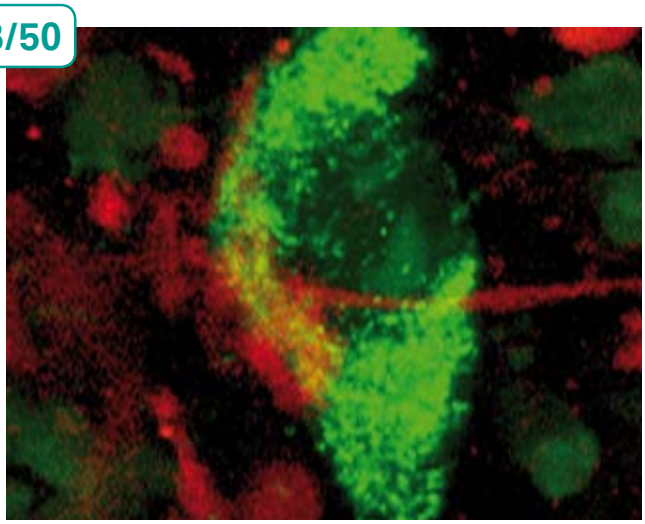

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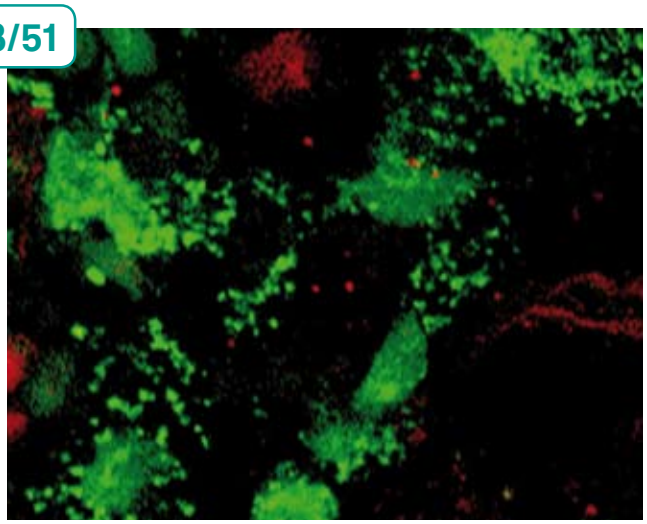

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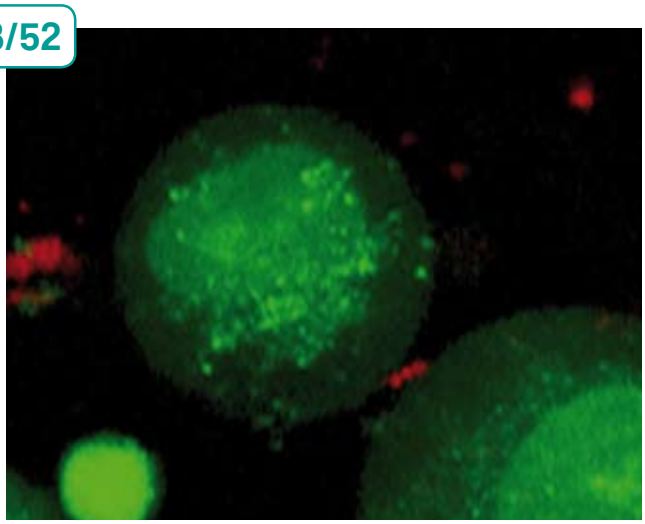

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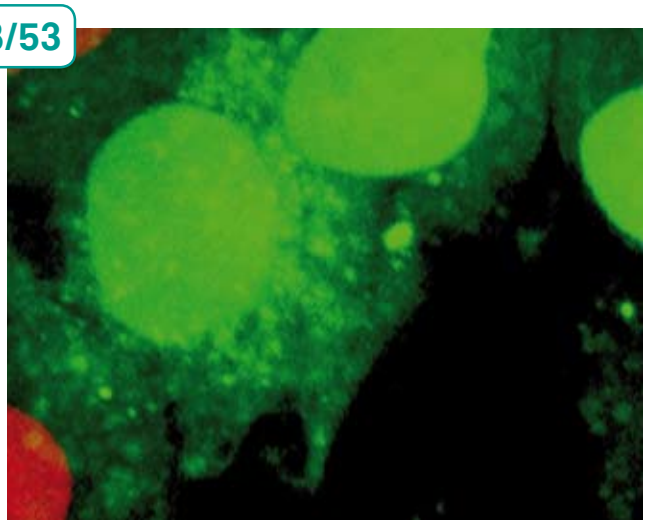

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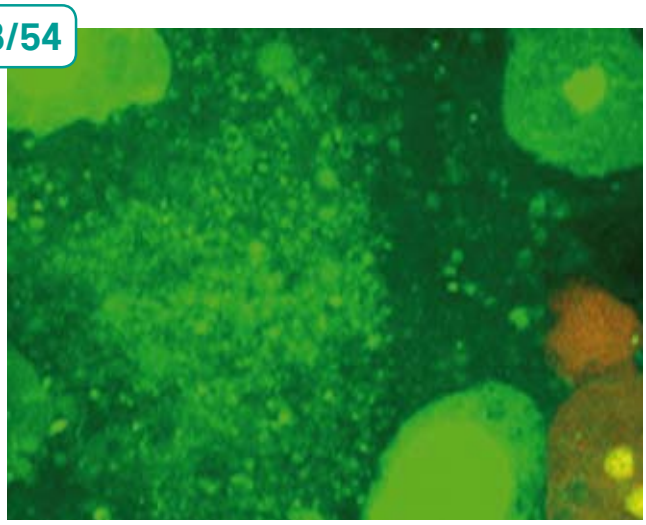

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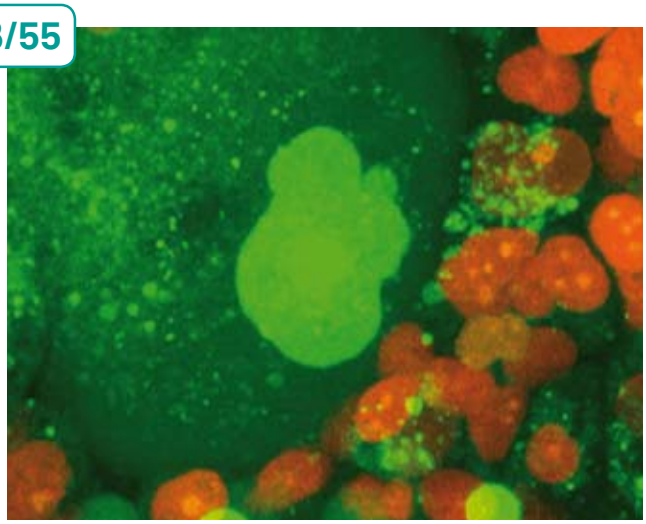

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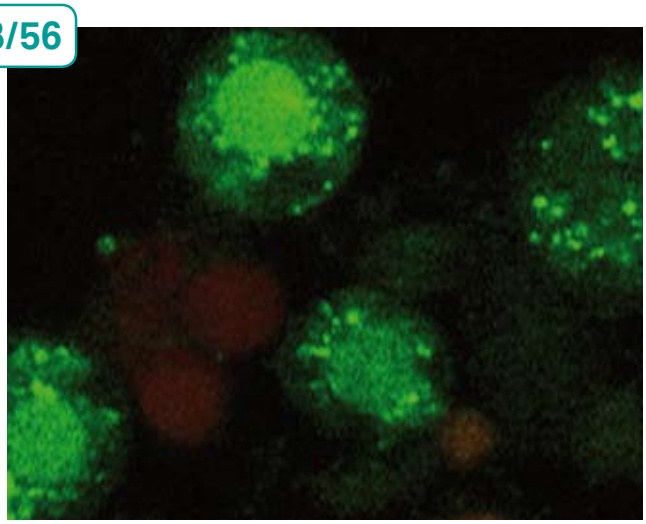

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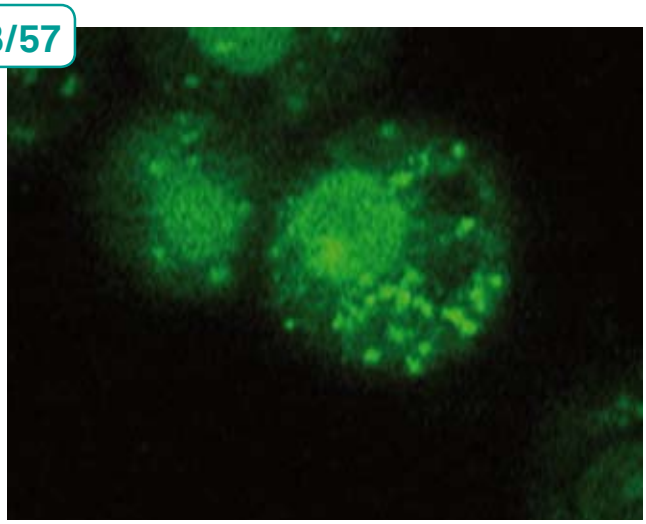

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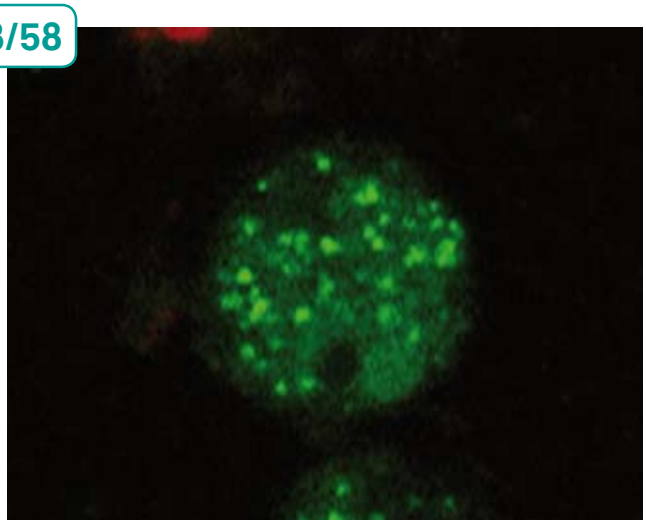
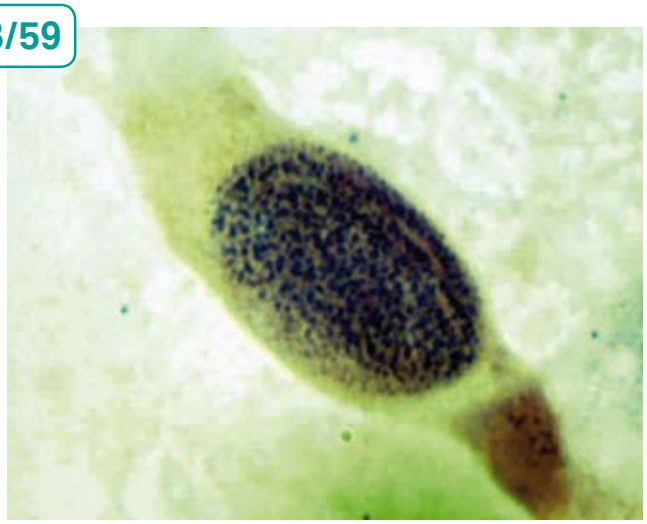


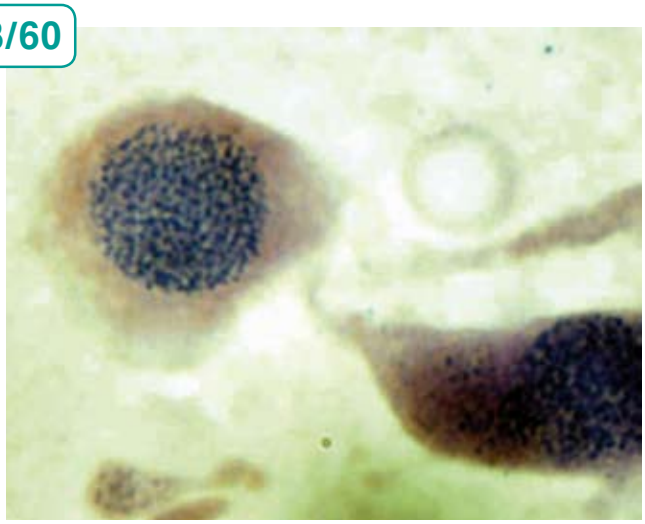
Crowded cocci inside nuclei of large cells (reproductive forms)
(×2000 Gram staining)

FIGURES 8/59 TO 8/69

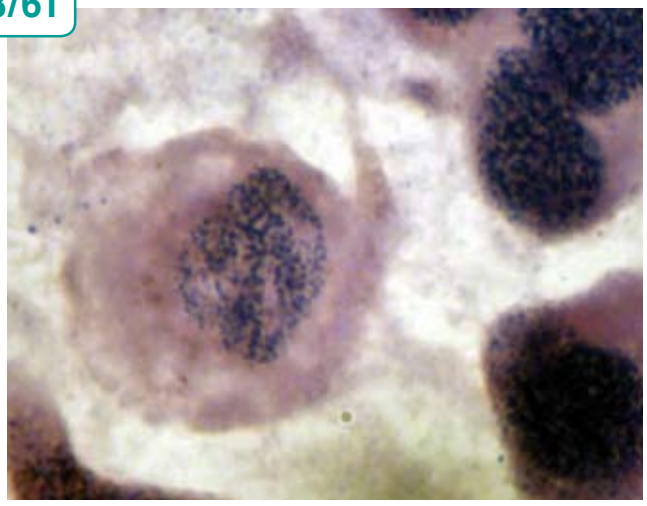
8/59



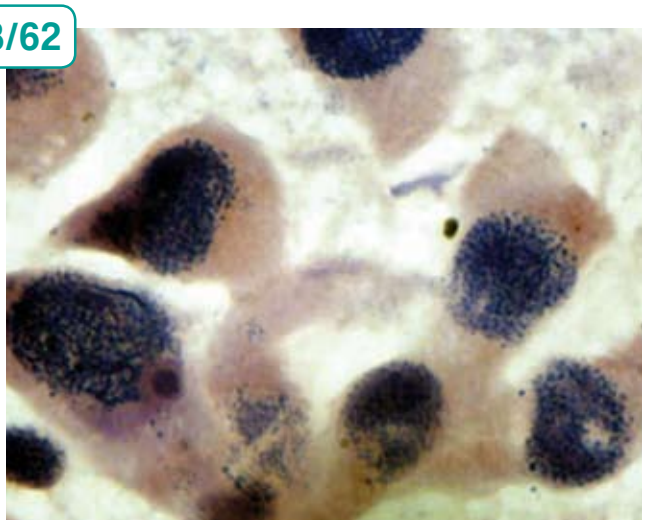
8/60



8/61



8/62



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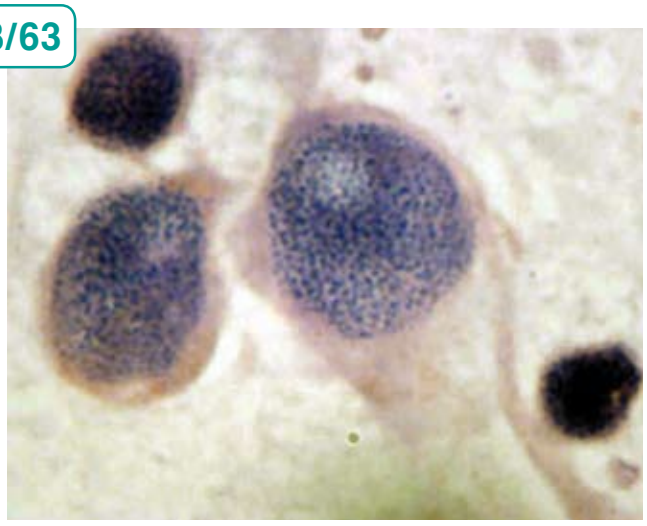
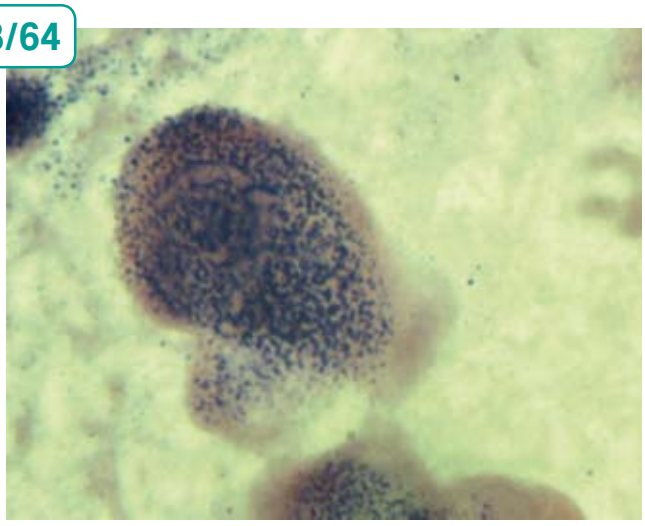


8/64



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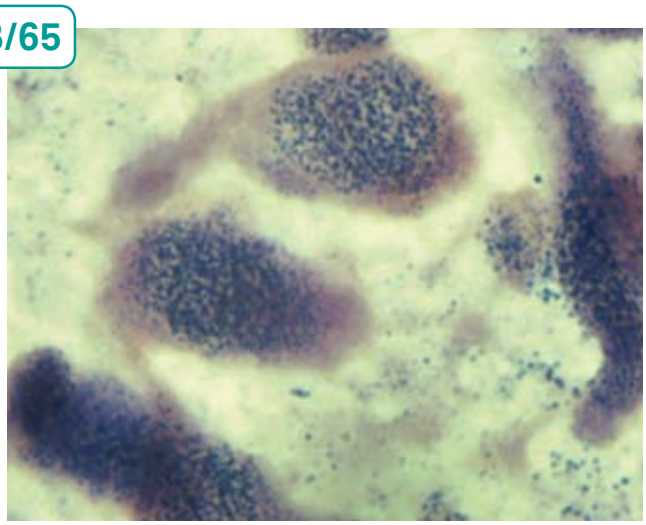
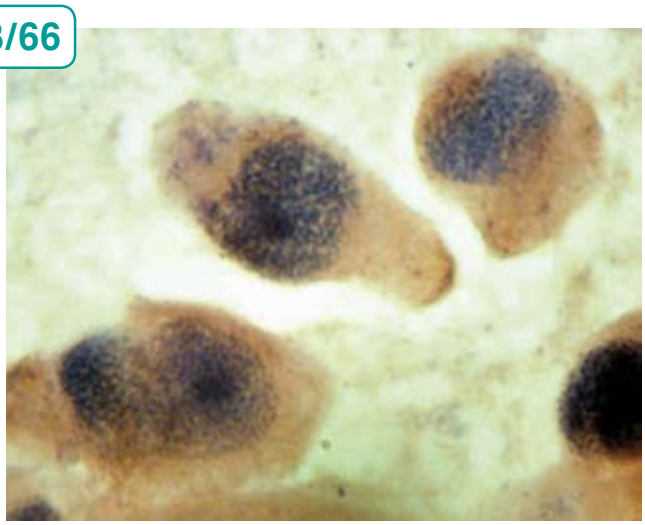
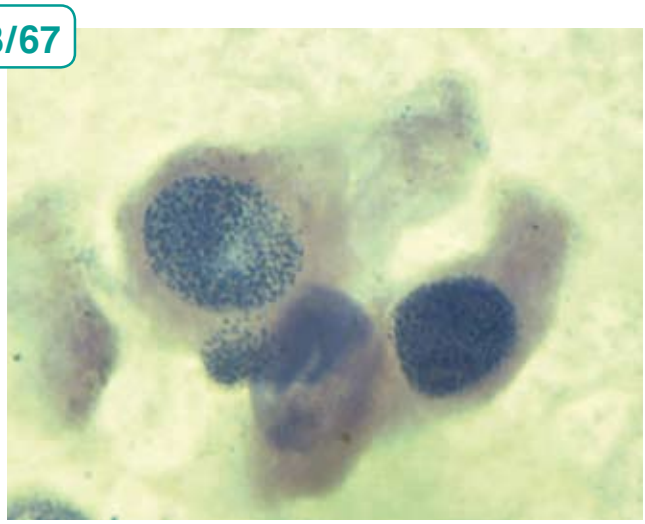


8/66



8/67



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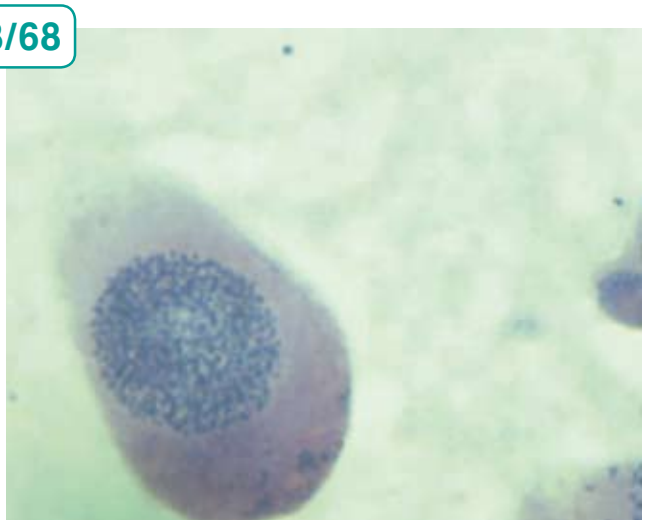

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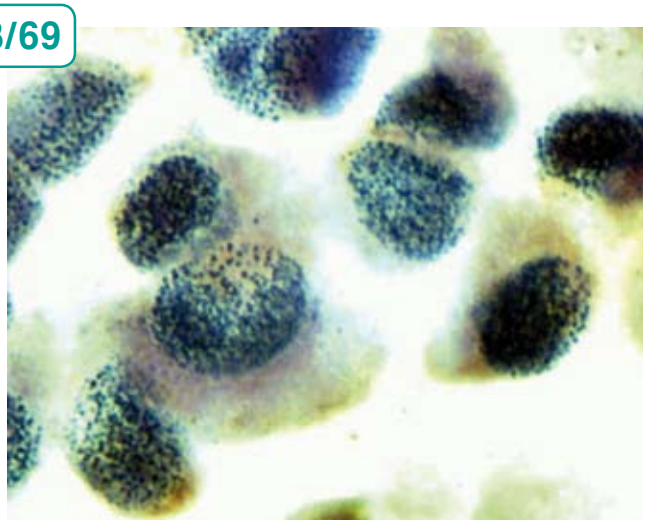
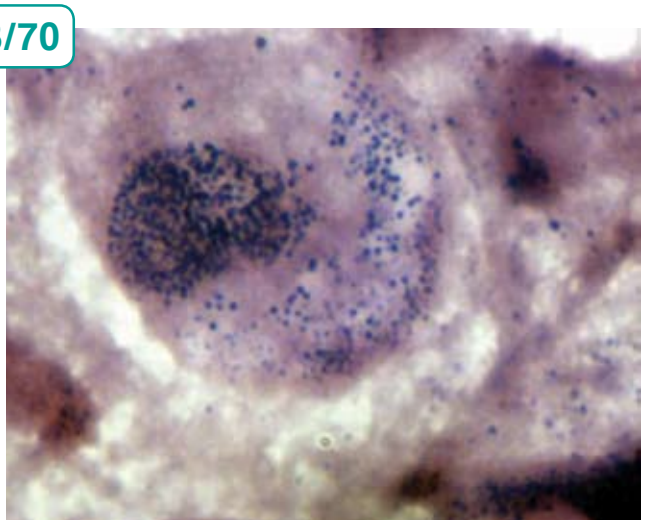


Extranuclear cocci are larger than intranuclear cocci, when they leave tumor cells (infective forms) (×2000 Gram staining)

FIGURES 8/70 TO 8/87

8/70



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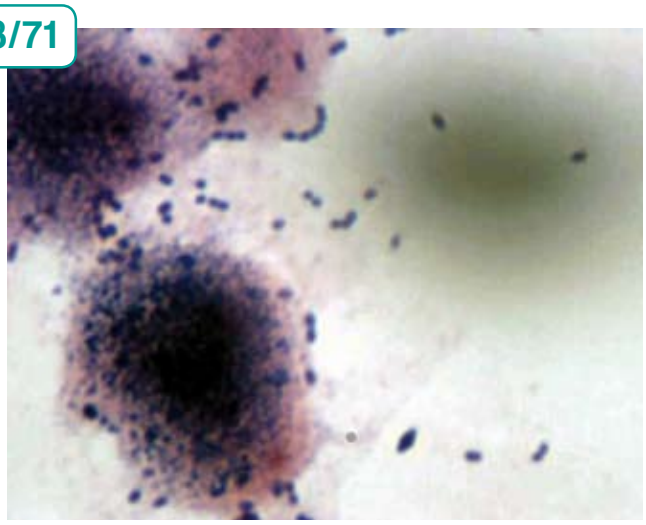
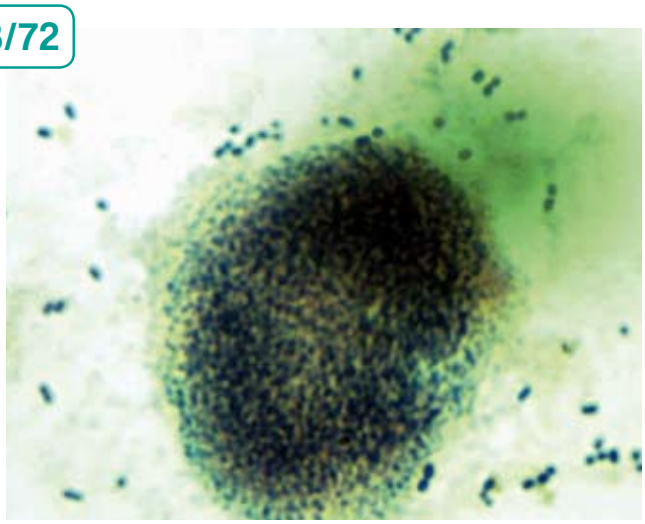


8/72



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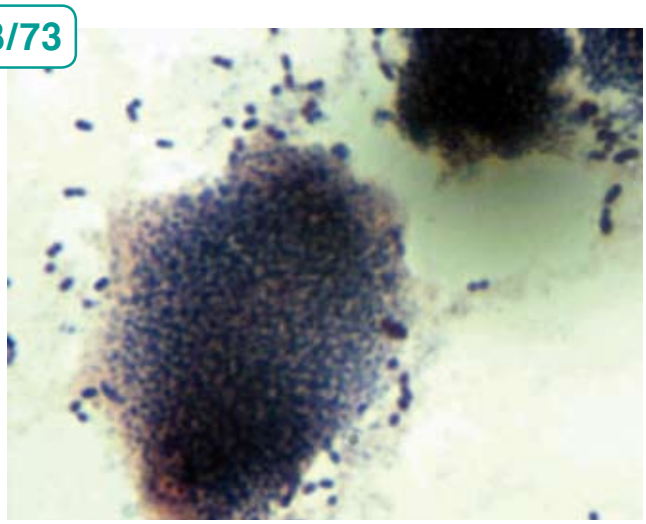

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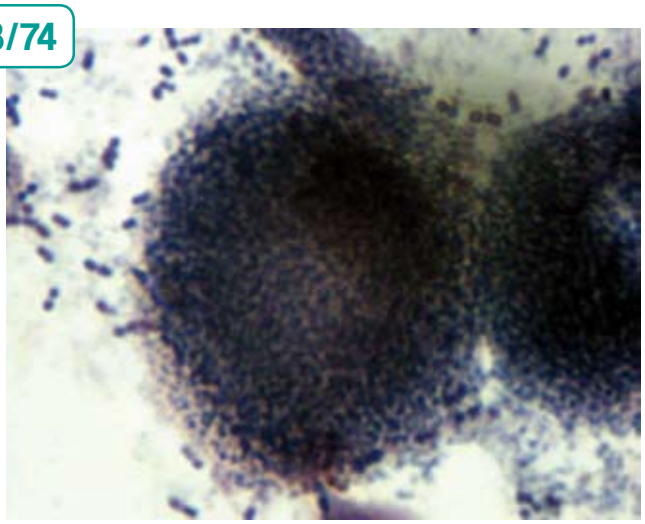
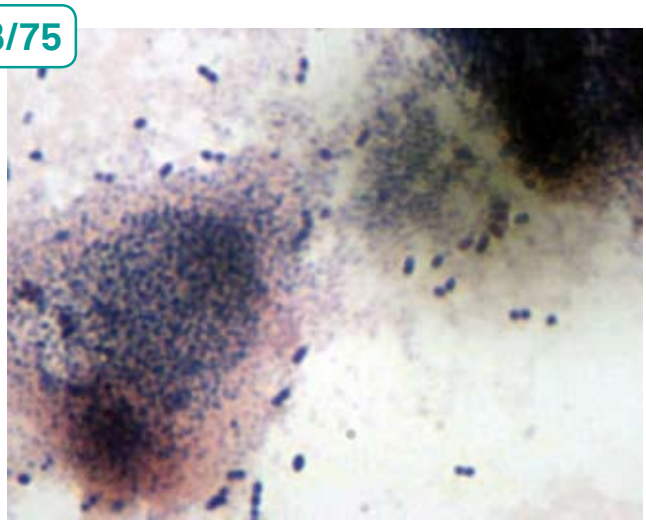
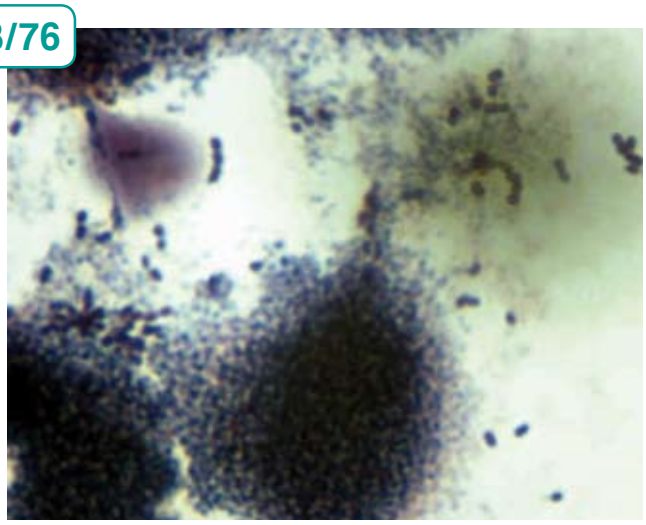


8/75



8/76



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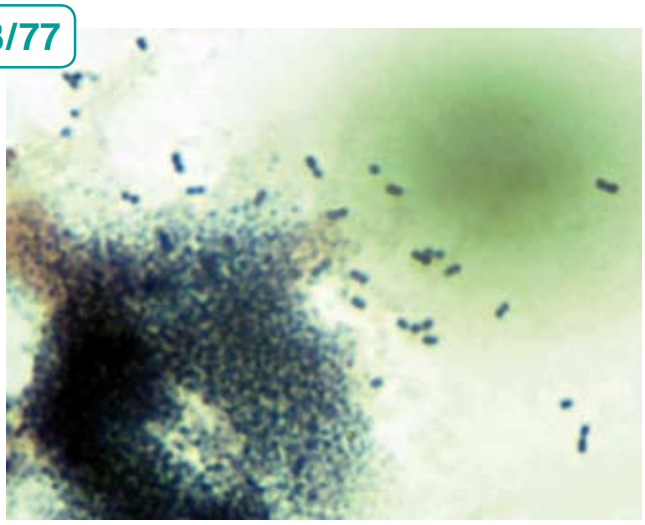
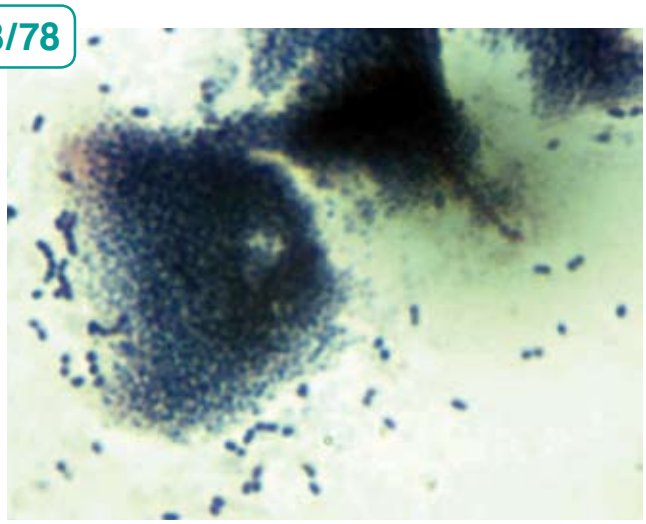


8/78



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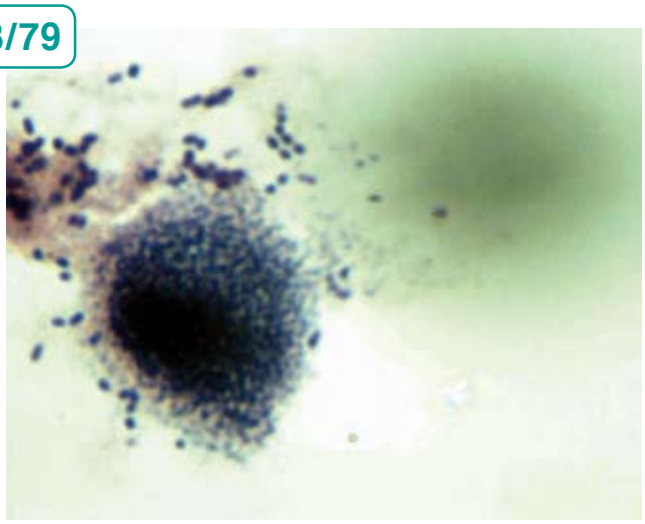

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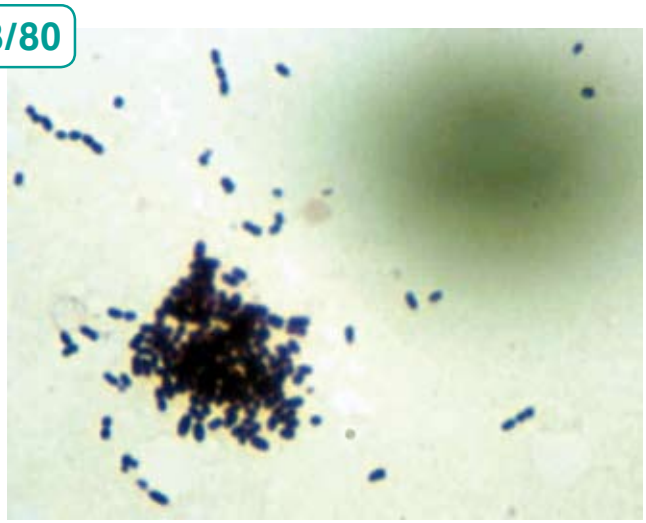
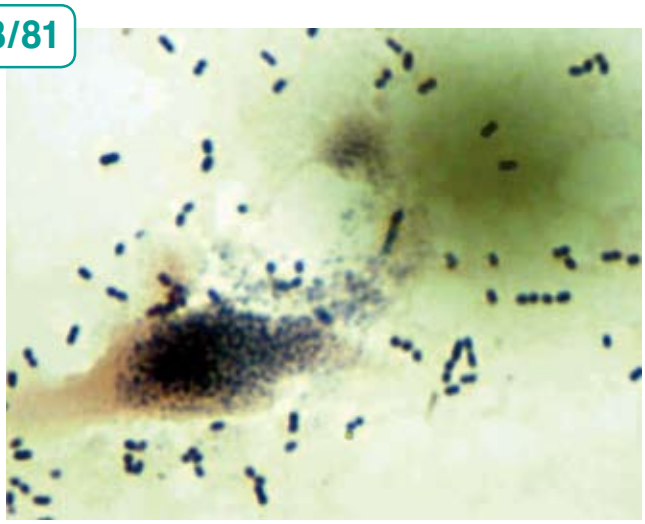
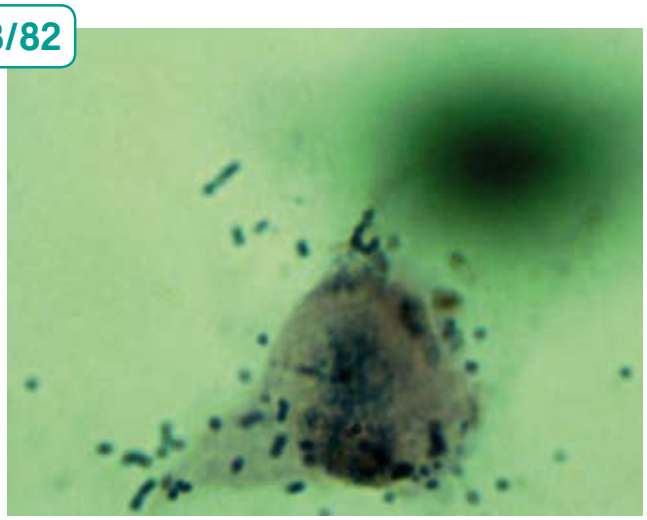


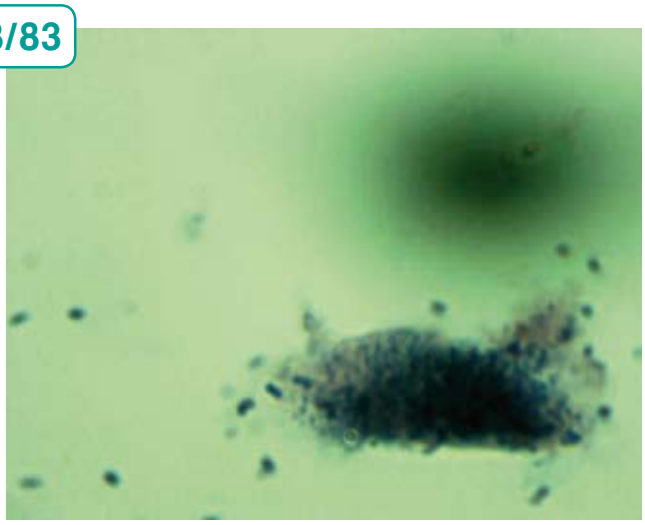
8/81



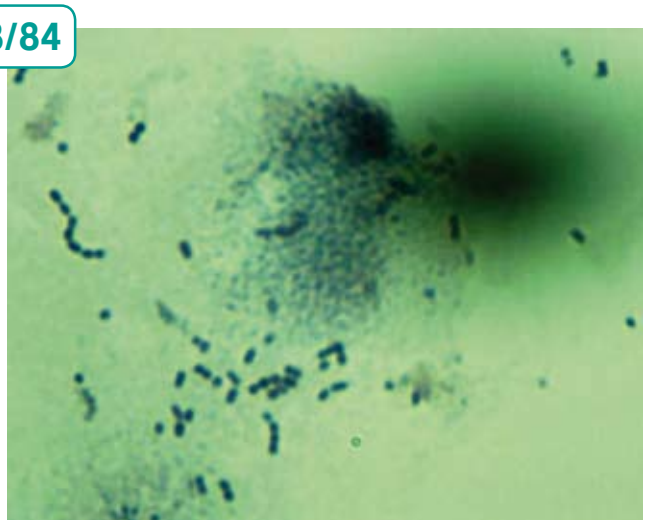
8/82



8/83



8/84



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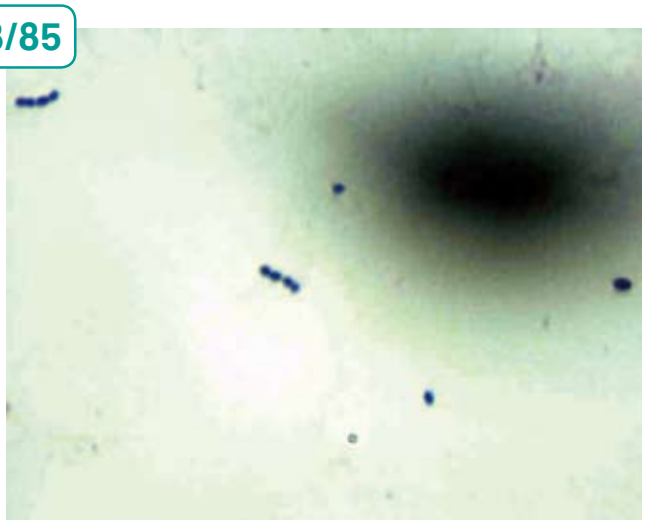
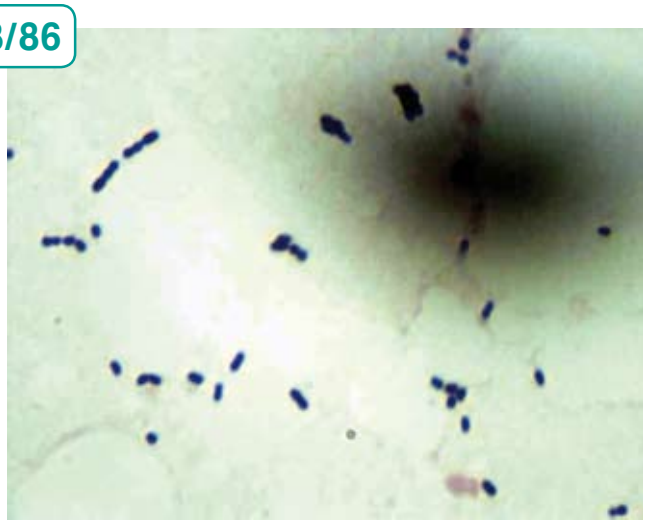


8/86



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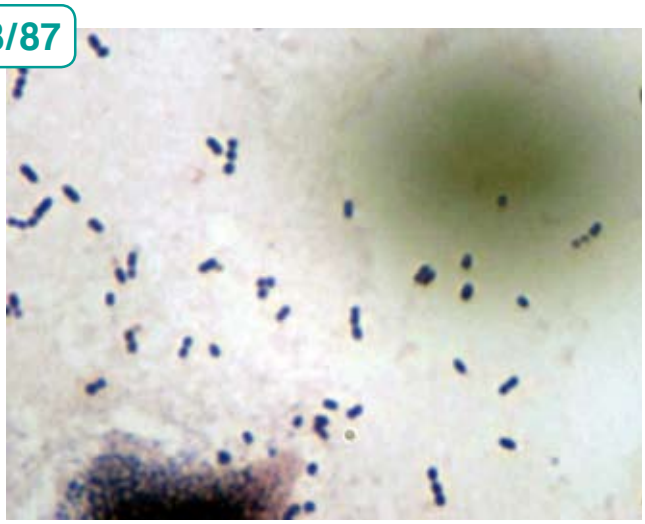
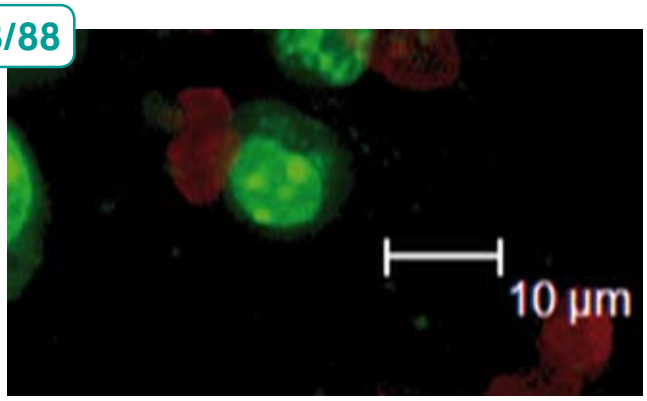


Syto 9 plus Propidium iodide staining

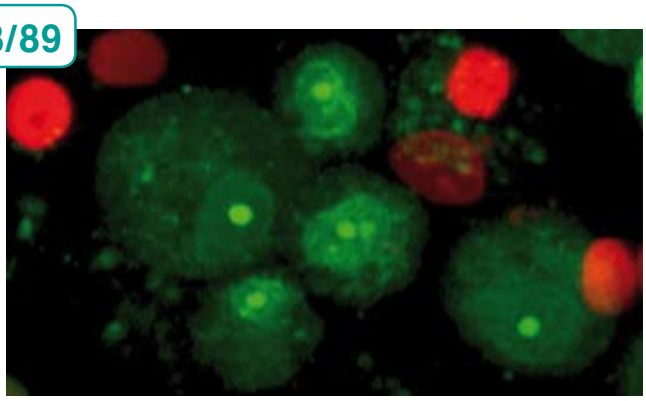
FIGURES 8/88 TO 8/148

→ **New nuclei: size variation are among 1 to >10 µm** (Figures **8/88** to **8/94**)

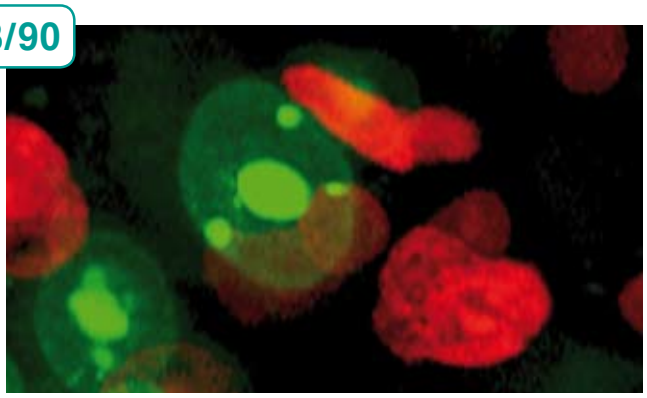
8/88



8/89



8/90



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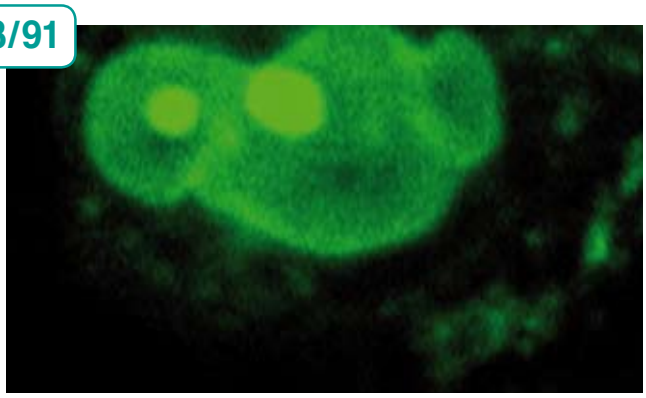

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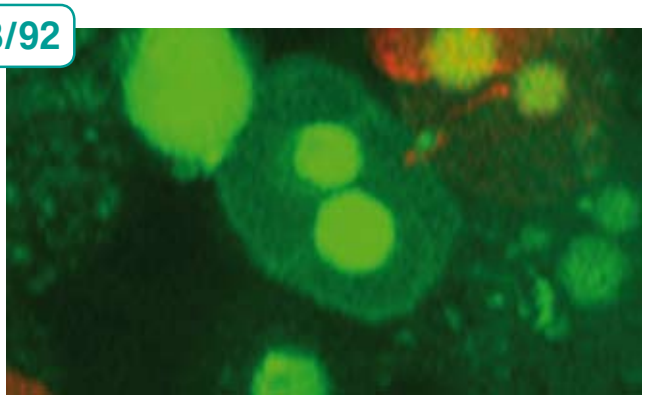

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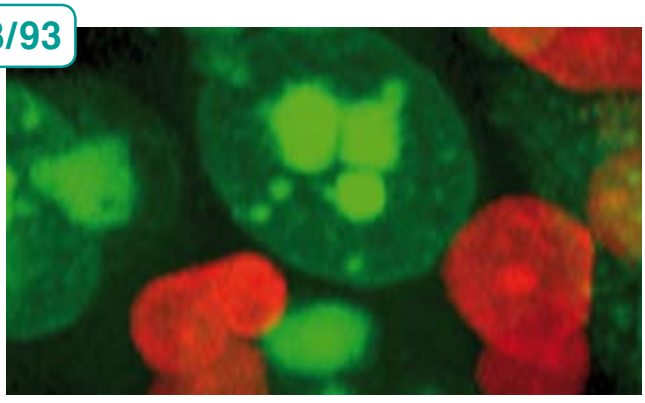
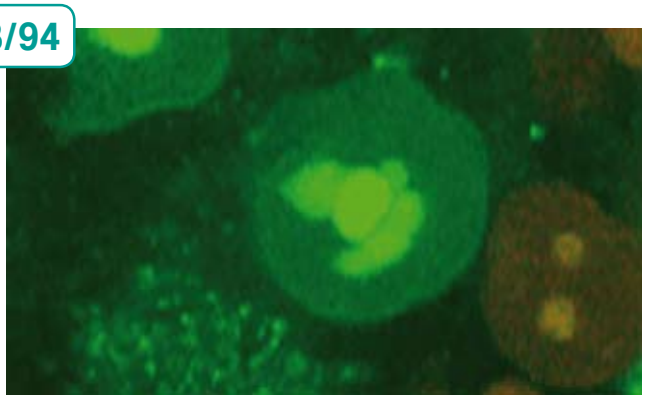
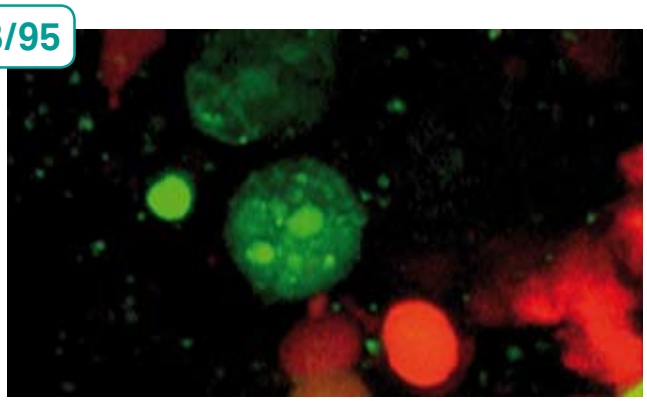


8/94

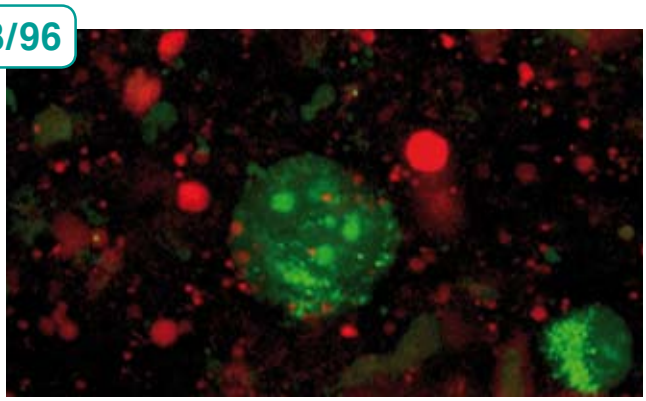


→ **New nuclei: examples of round morphologies, when the nuclei are several inside cells they seem a "balloon release"** (Figures **8/95** to **8/98**)

8/95



8/96



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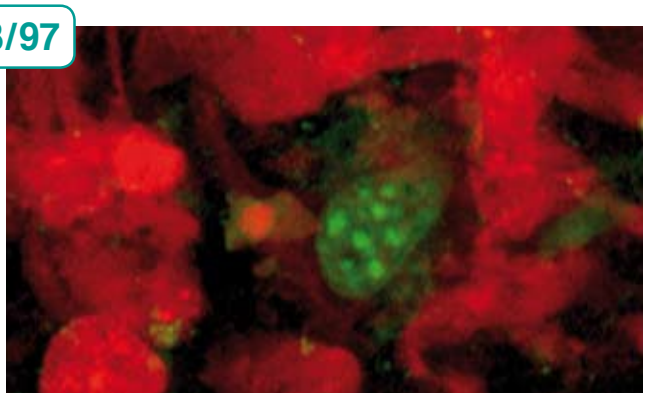
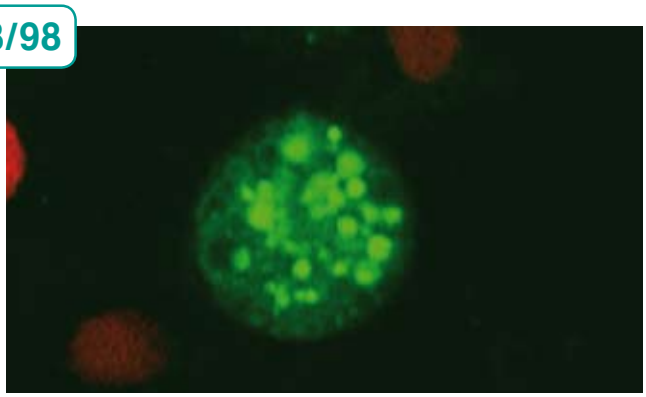
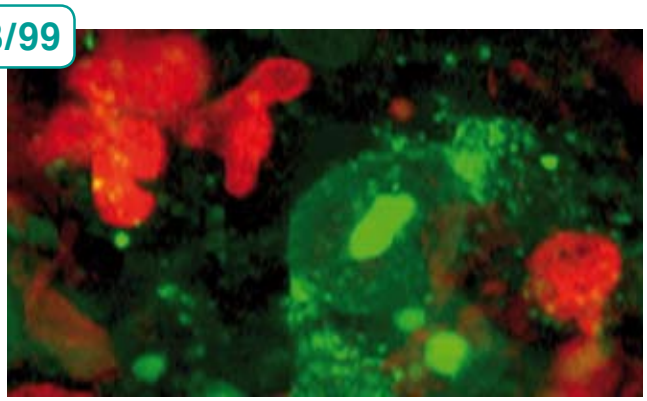


8/98

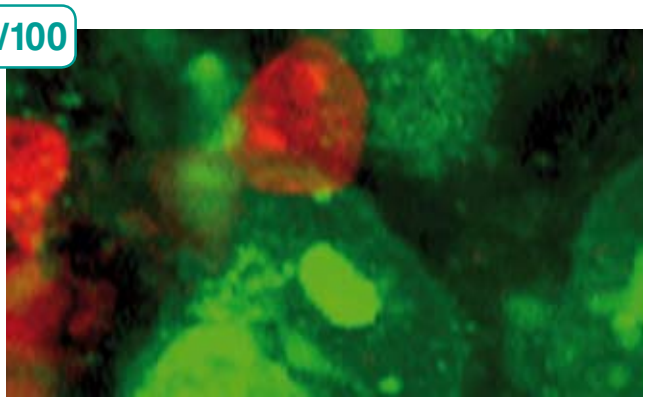


→ **New nuclei: examples of stick-like morphology (rods)** (Figures **8/99** to **8/104**)

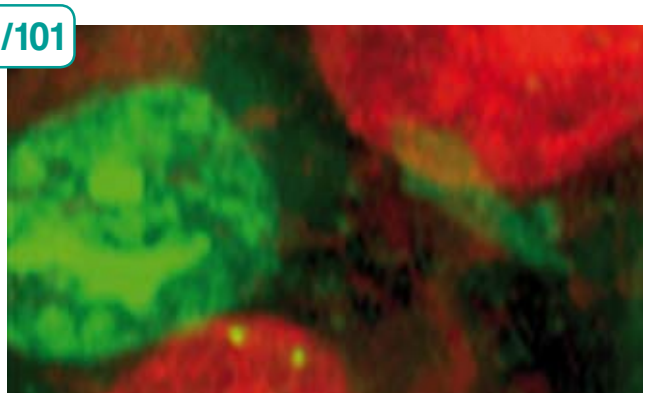
8/99



8/100



8/101



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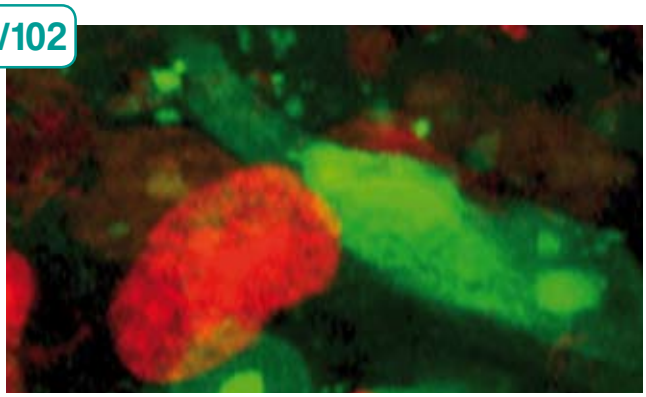

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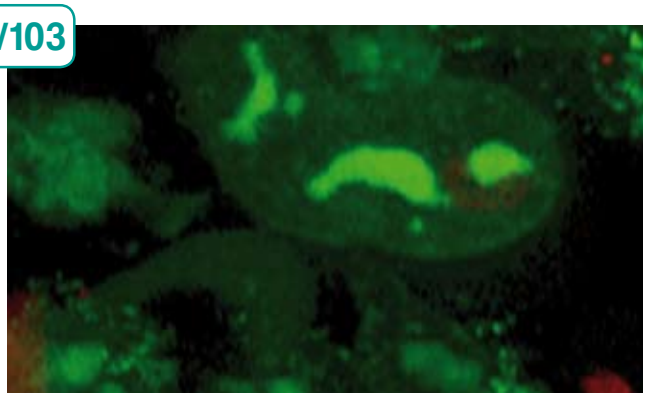
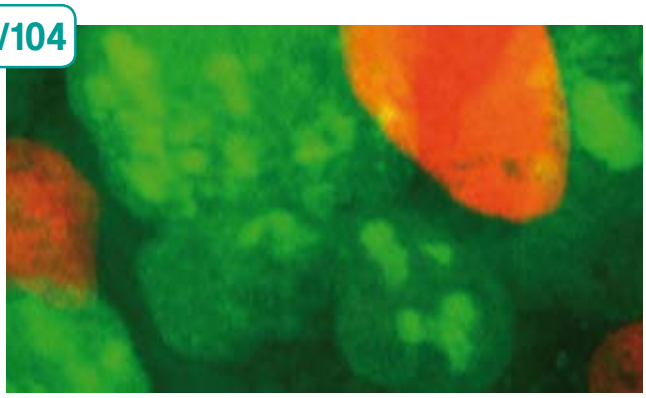
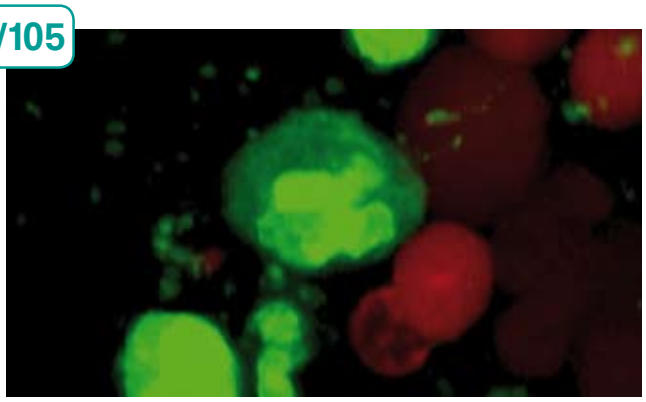


8/104



→ **New nuclei: examples of X-like morphology** (Figures **8/105** and **8/106**)

8/105



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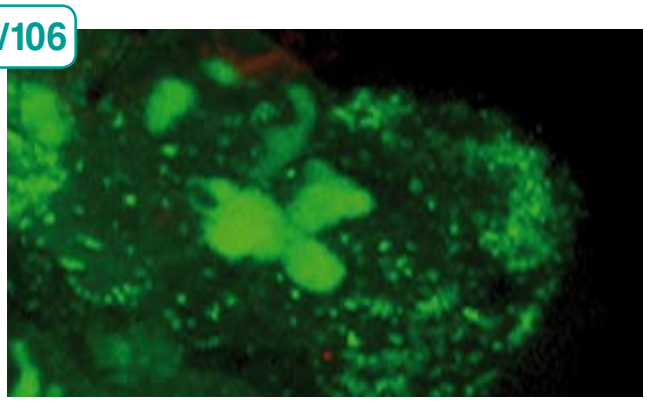

→ **New nuclei: examples of Y-like morphology** (Figures **8/107** and **8/108**)

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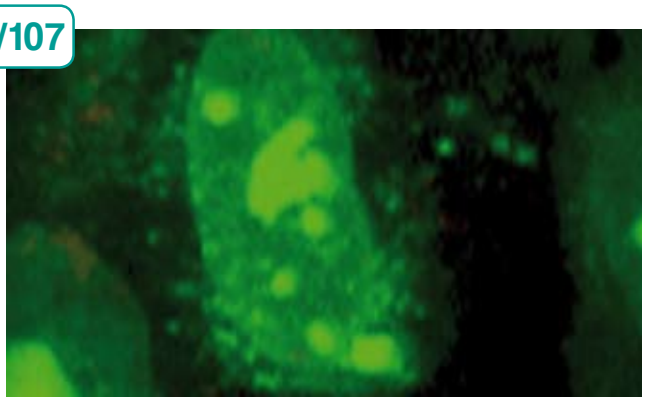
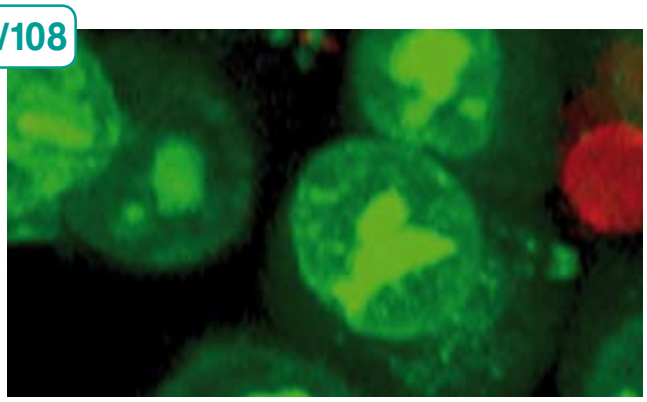
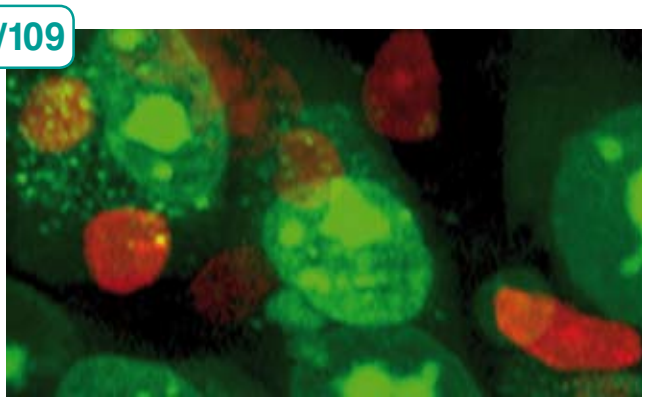


8/108

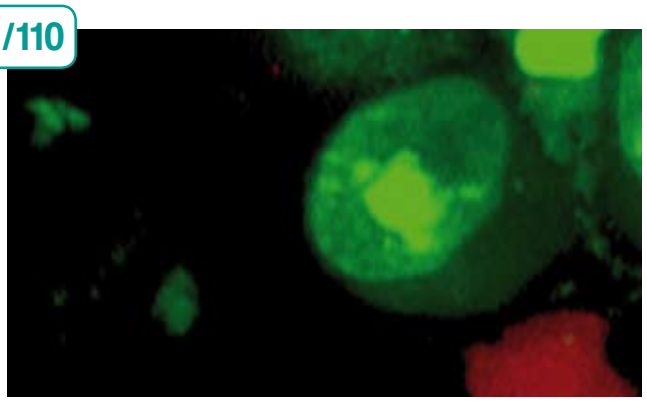


→ **New nuclei: examples of irregular morphology** (Figures **8/109** to **8/111**)

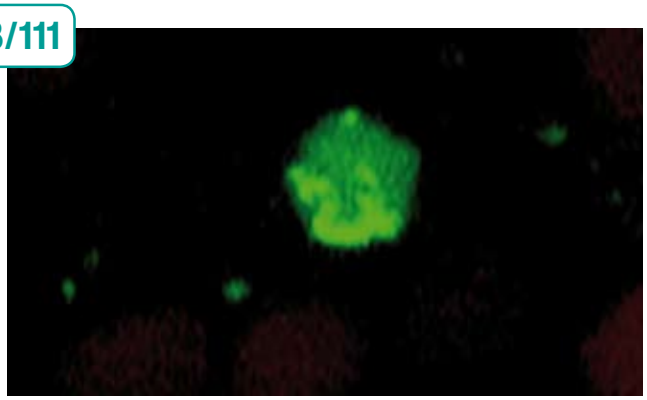
8/109



8/110

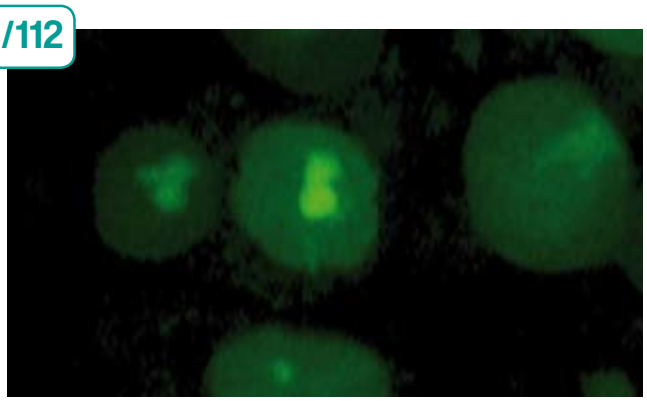


8/111

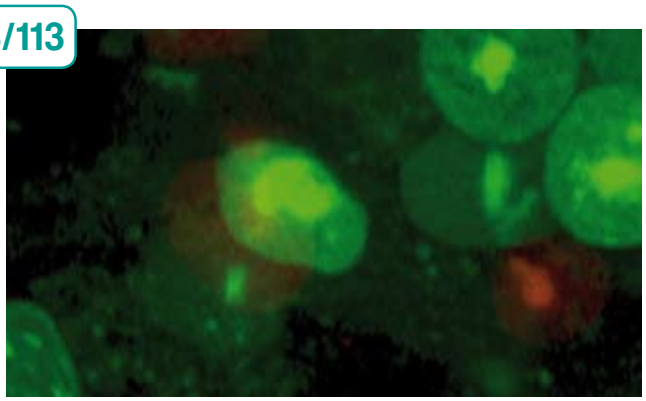


→ **New nuclei: examples of bipolar division** (Figures **8/112** to **8/117**)

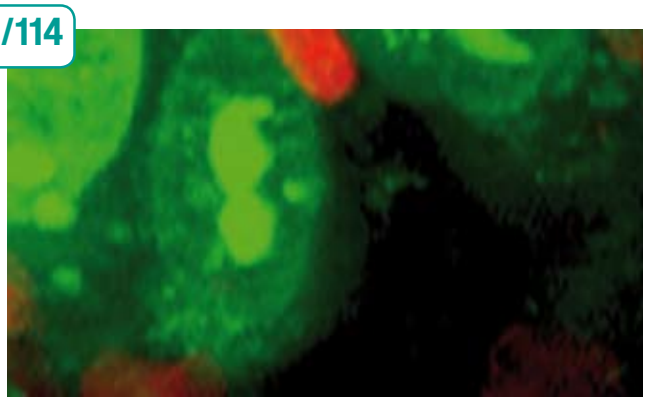
8/112



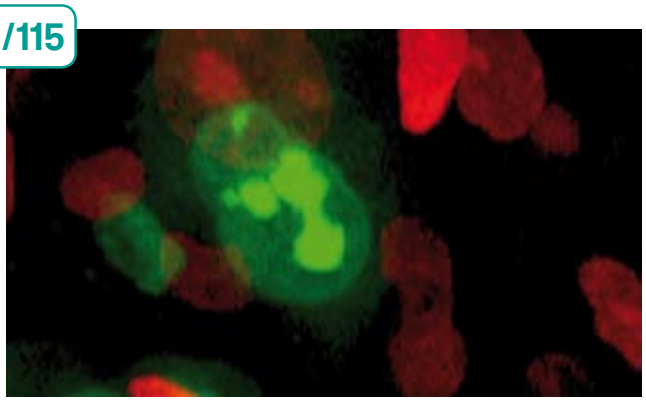
8/113



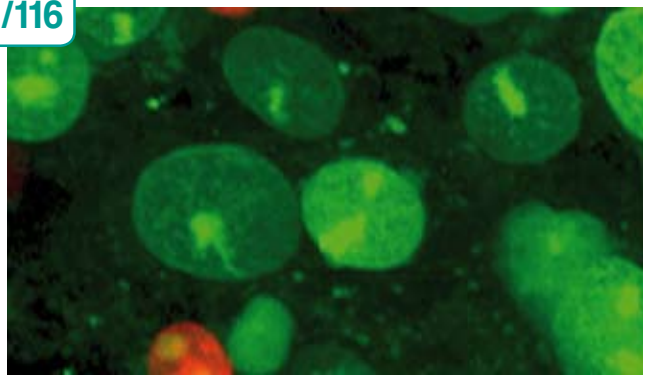
8/114



8/115



8/116



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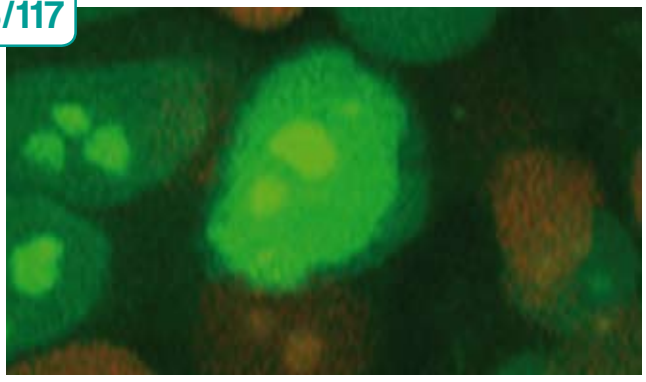
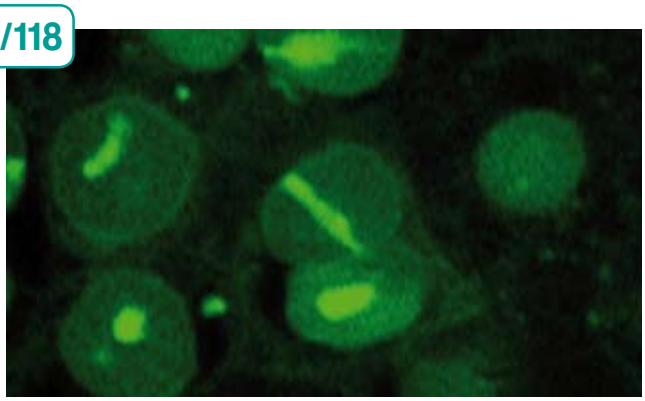
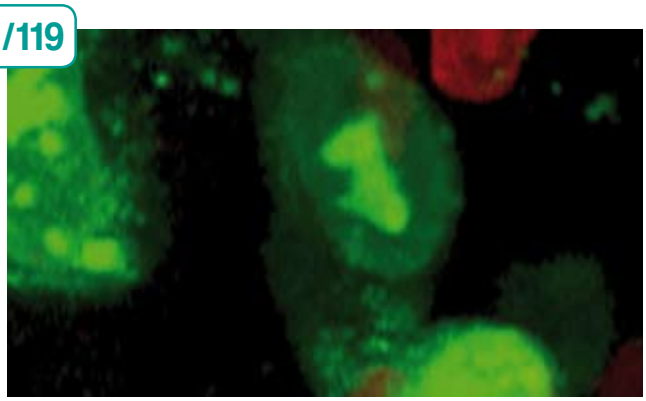


→ **New nuclei generation by several narrowings of long rods** (Figures **8/118** to **8/129**)

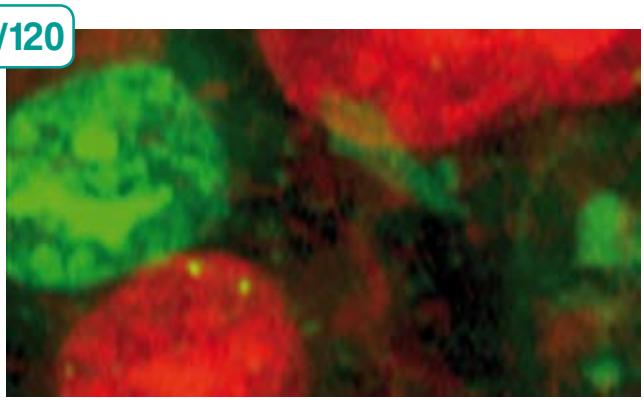
8/118



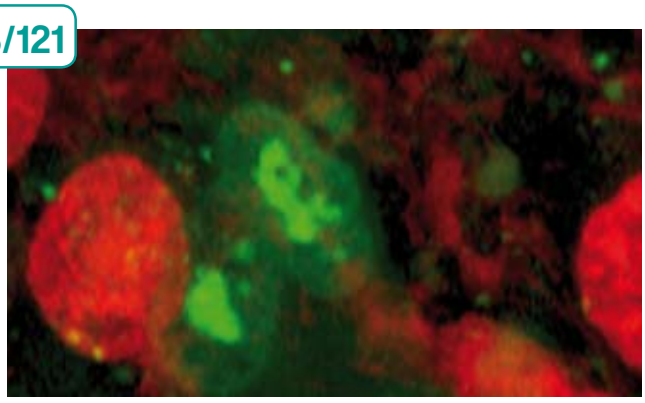
8/119



8/120



8/121



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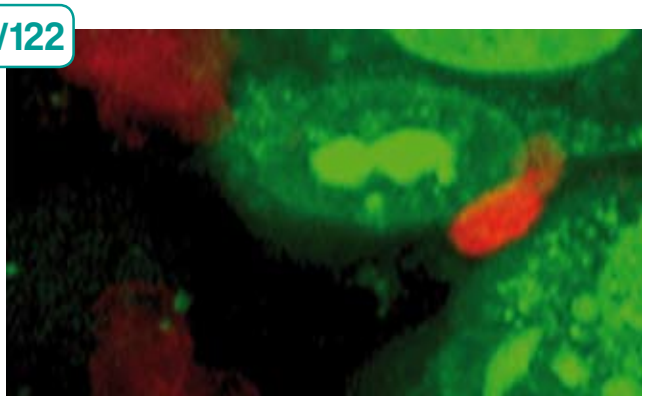
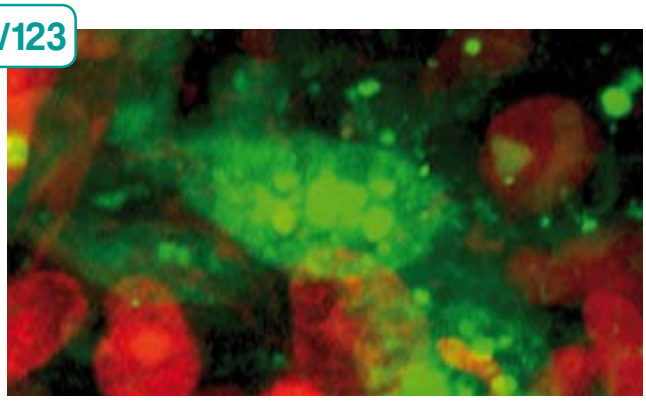
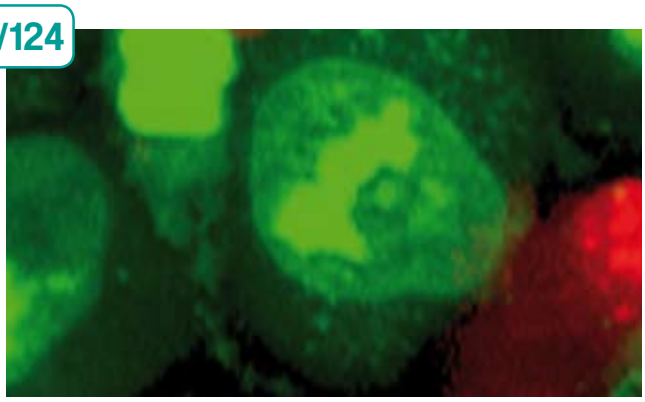


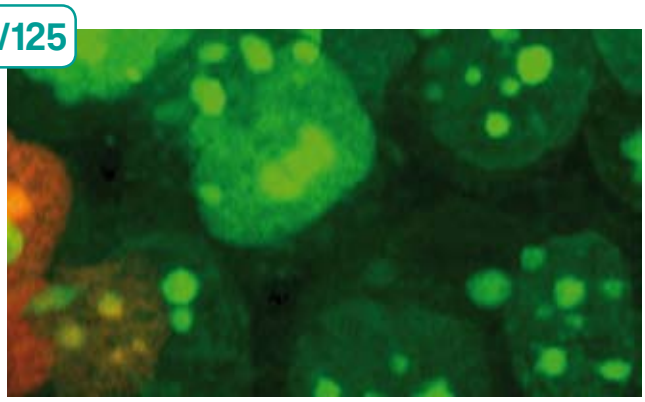
8/123



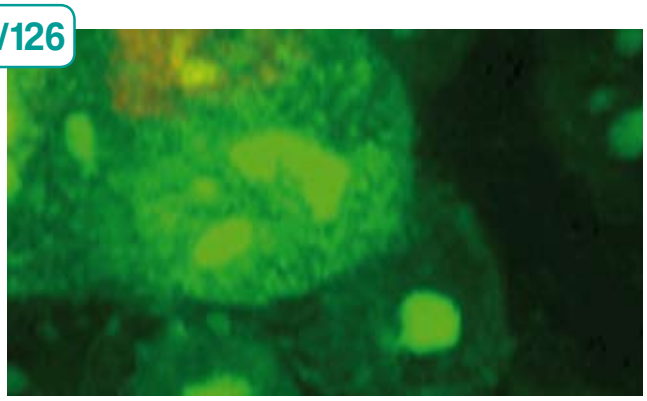
8/124



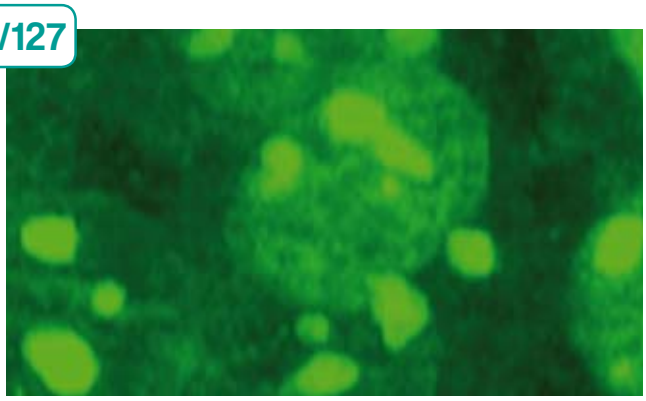
8/125



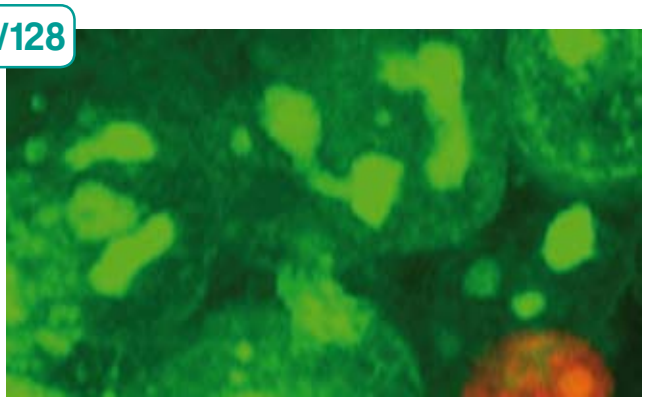
8/126



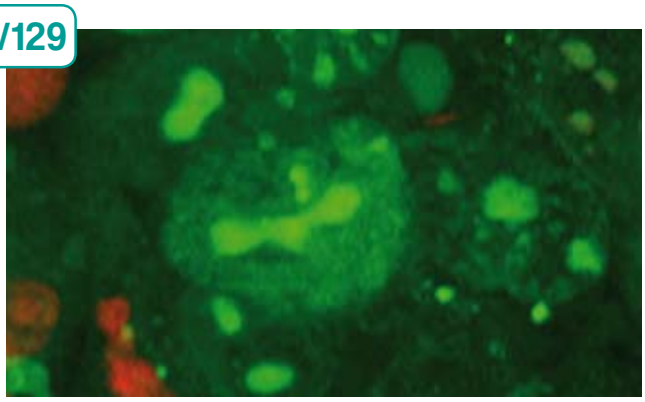
8/127



8/128



8/129



→ **New nuclei generation by narrowings and divisions of multiple close rods** (Figures **8/130** to **8/140**)

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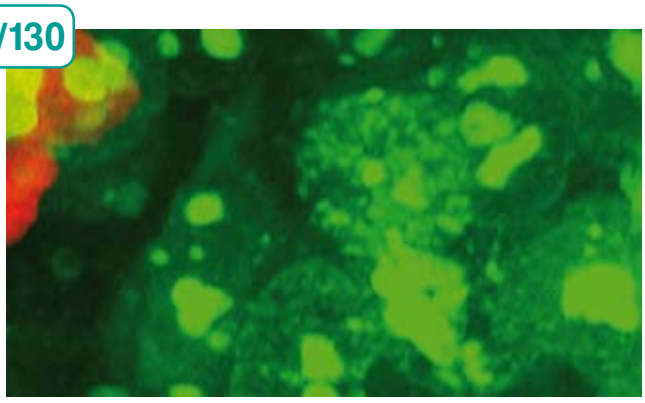
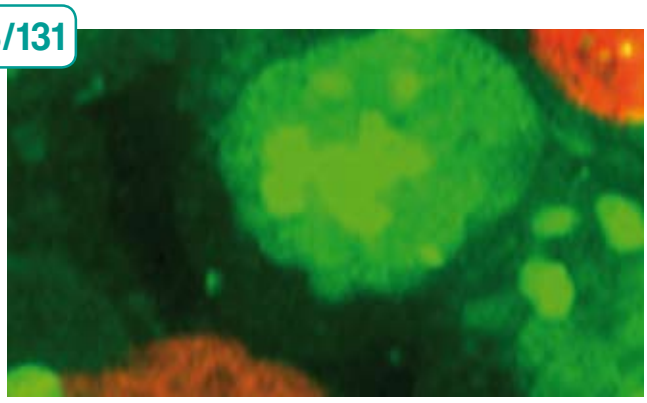
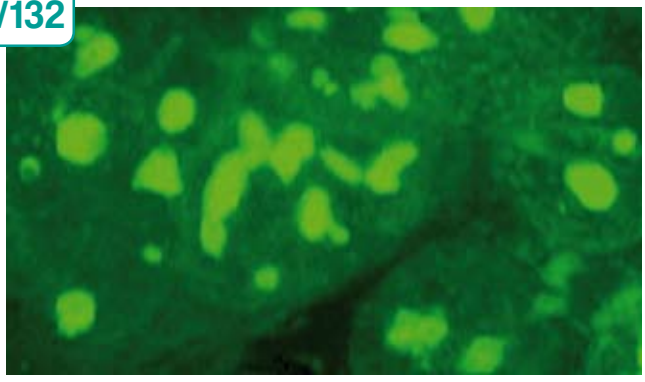


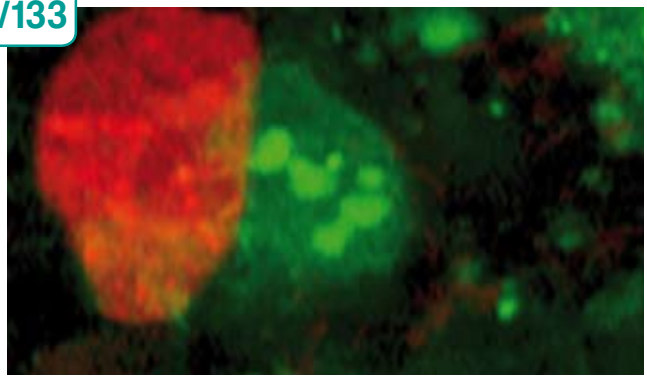
8/131



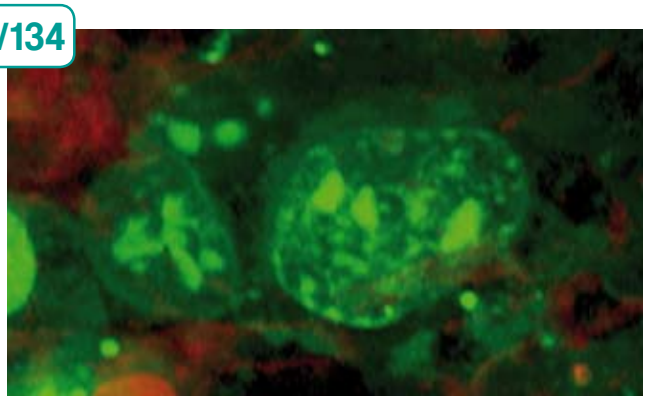
8/132



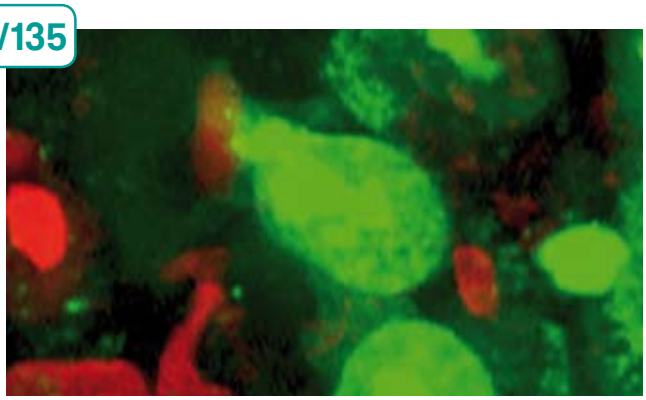
8/133



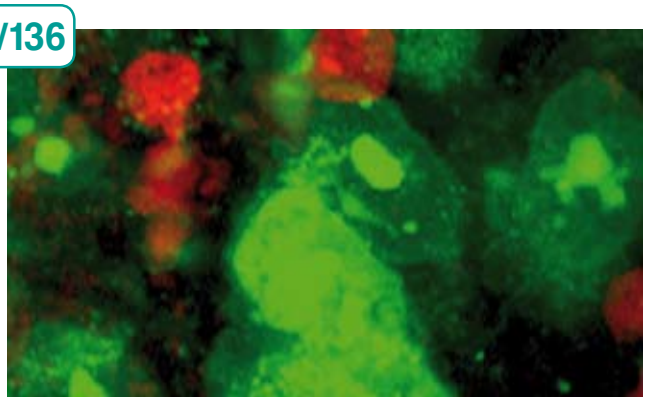
8/134



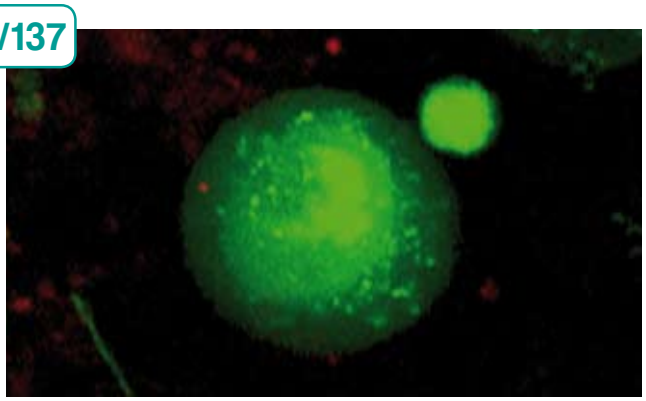
8/135



8/136



8/137



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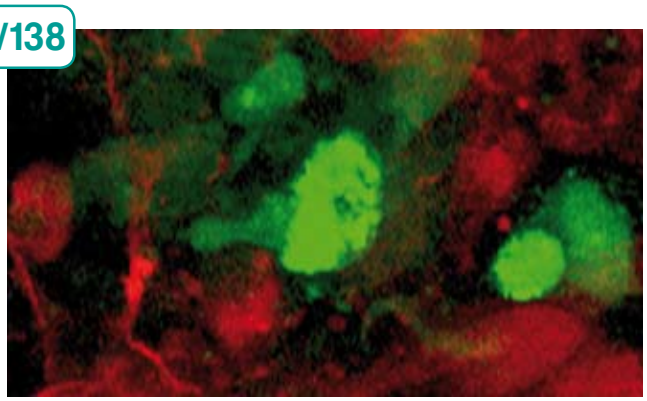

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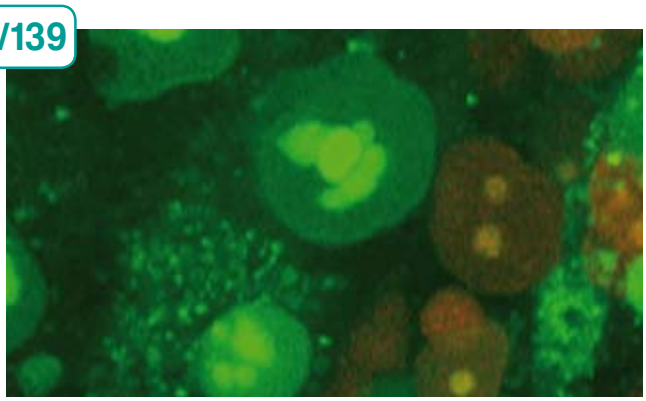

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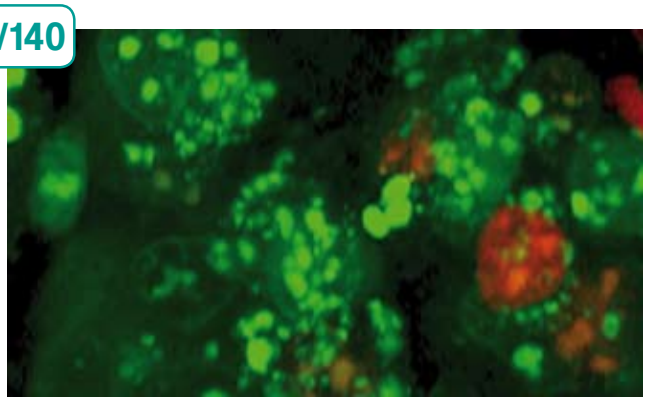

→ **New nuclei generation by multipolar divisions and "balloon release" distribution inside cells**
(Figures **8/141** to **8/148**)

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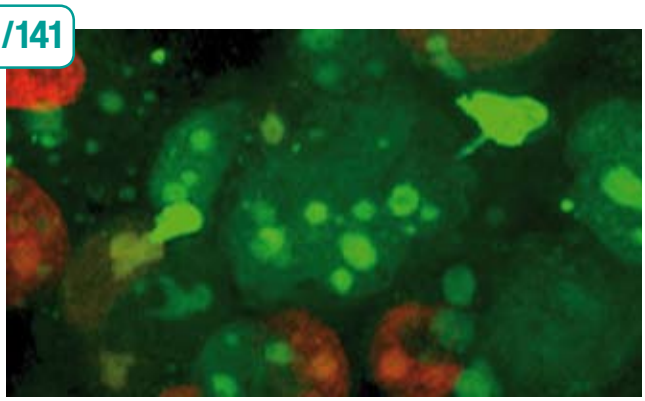

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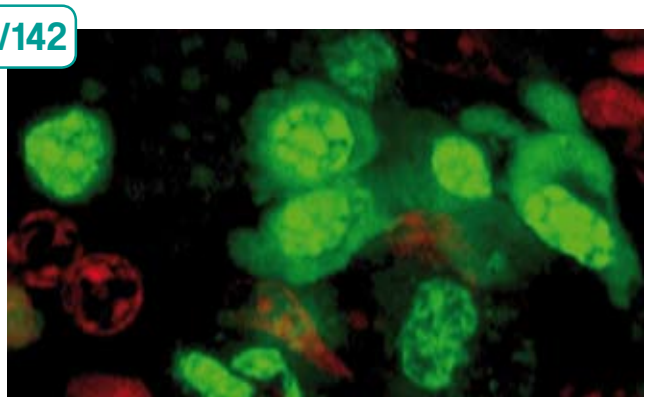

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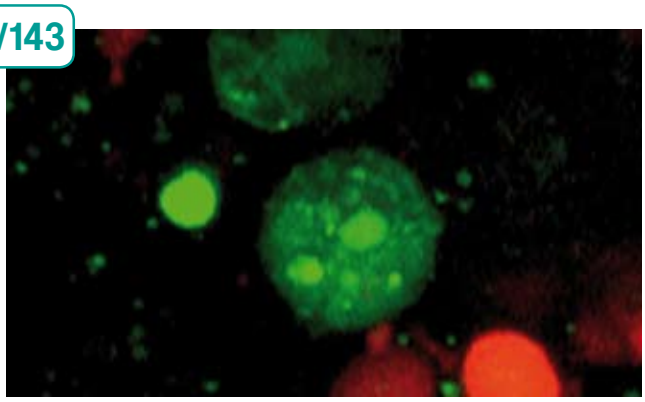

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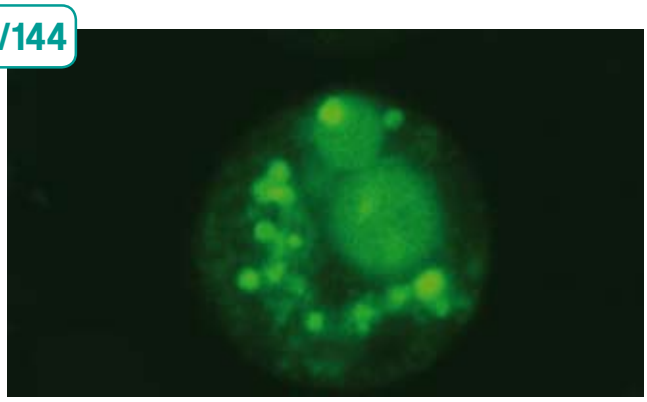

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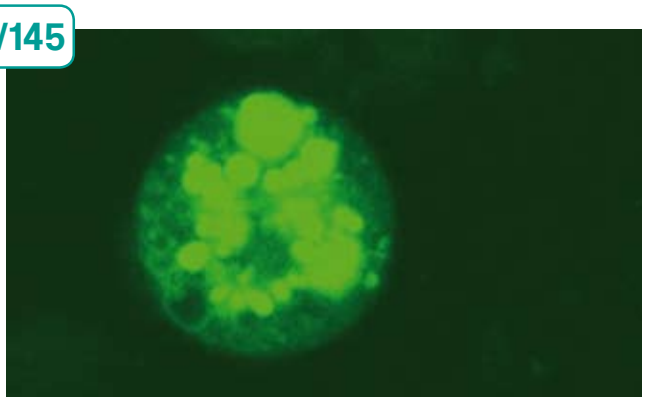

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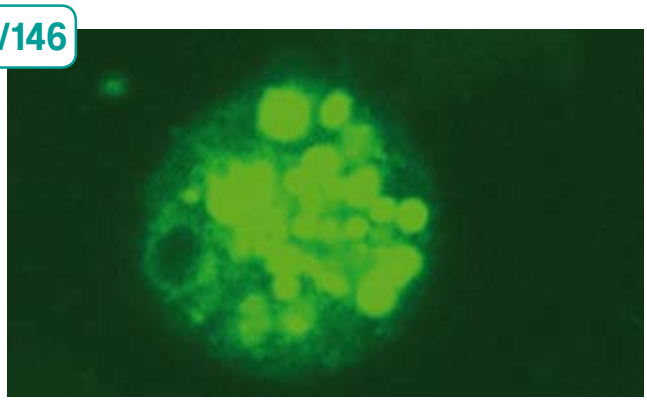

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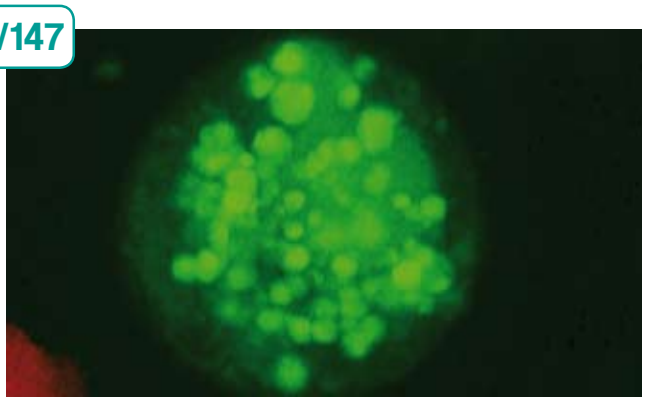

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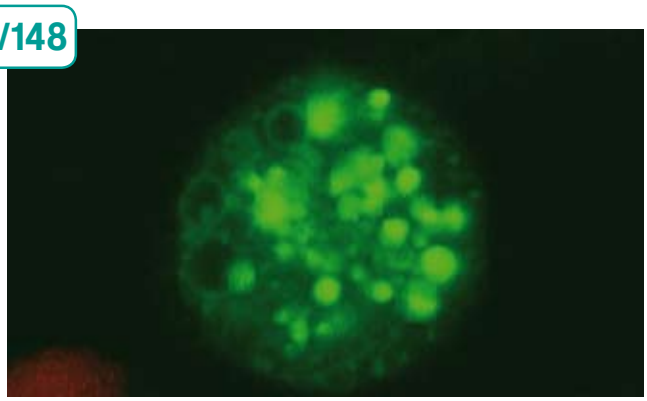

➔ **New nuclei bipolar division, showing cocci distributed between daughter cells** (reproductive forms) (×2000, Gram staining) (Figures **8/149** to **8/162**)

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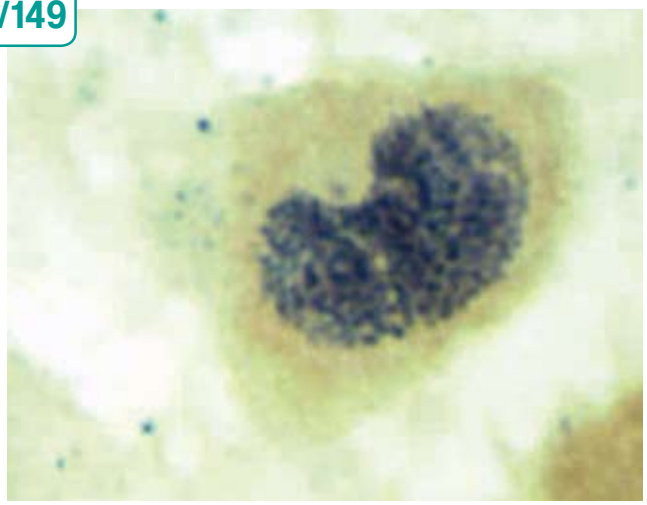

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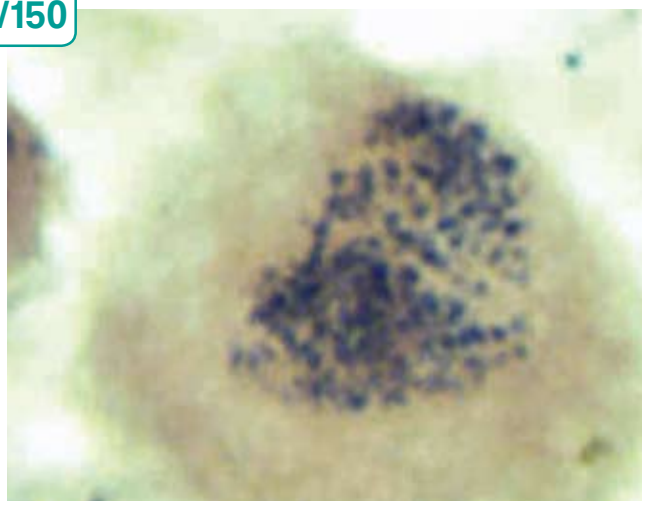

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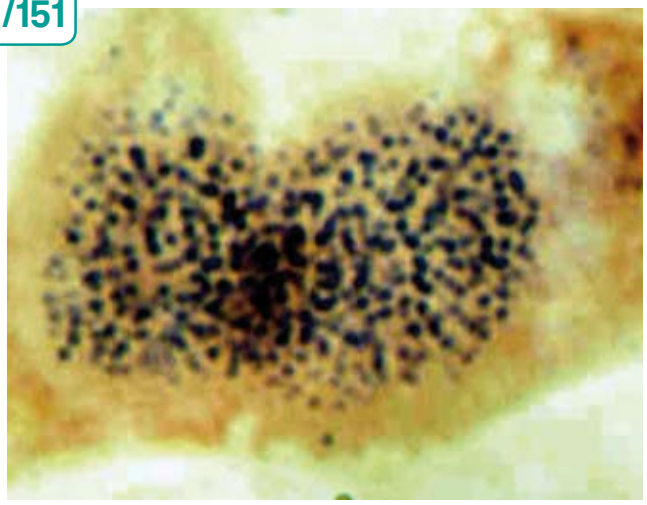

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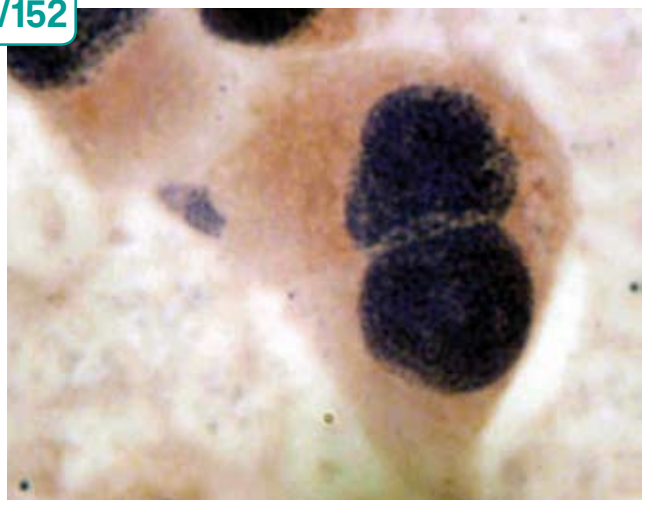

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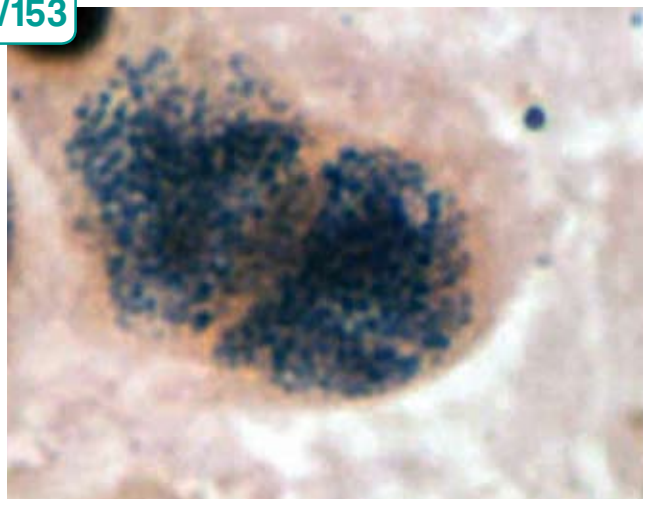

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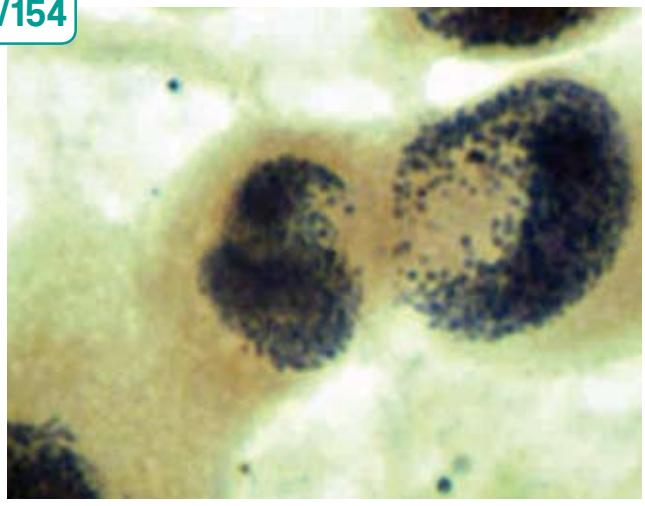

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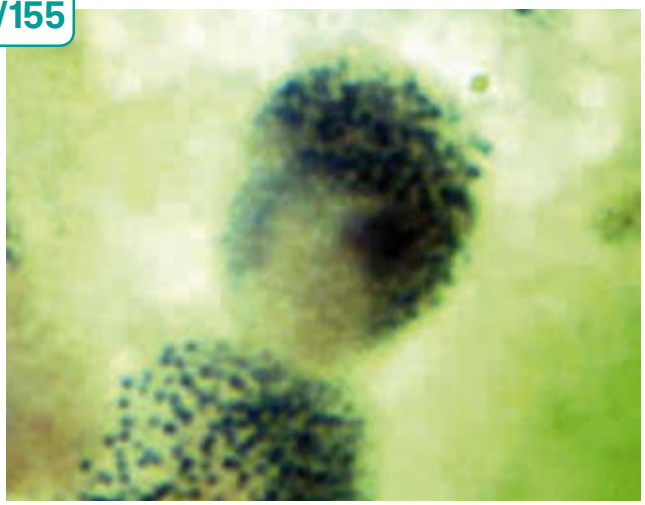

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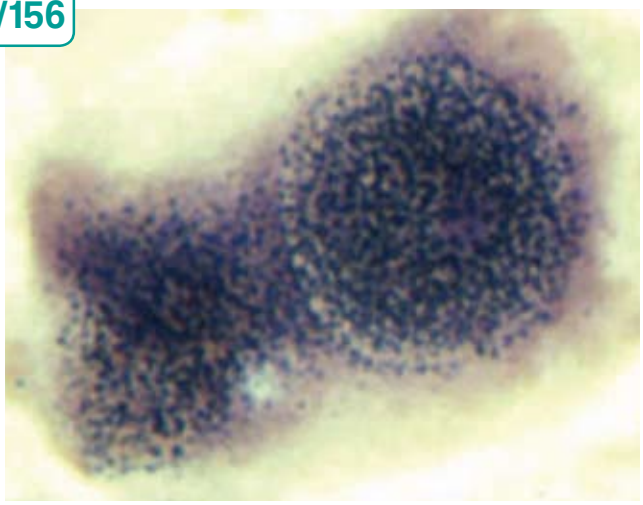

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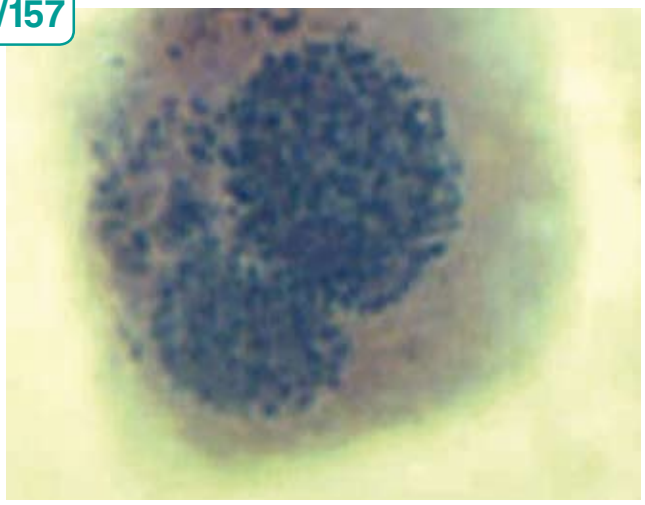

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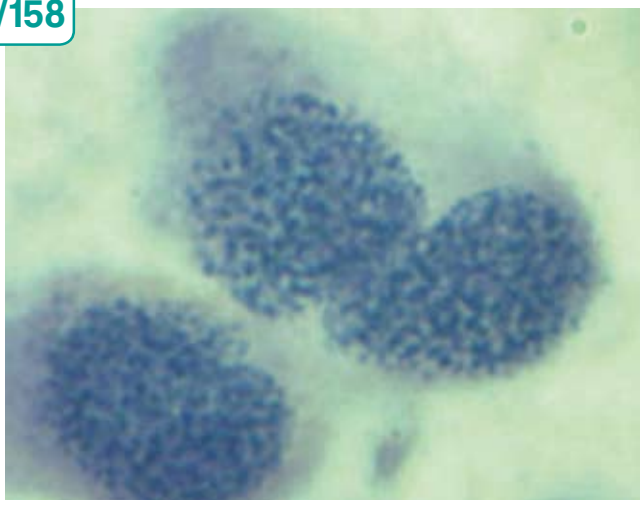

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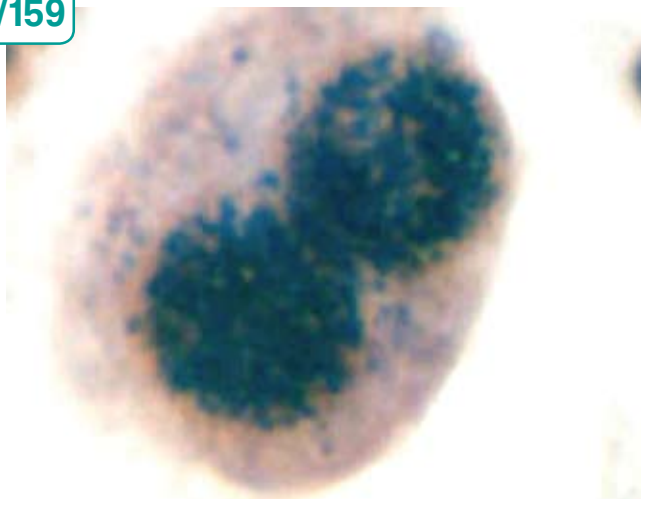

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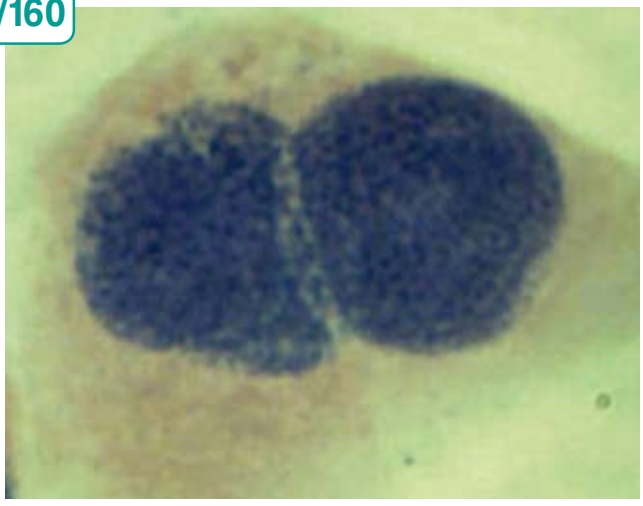
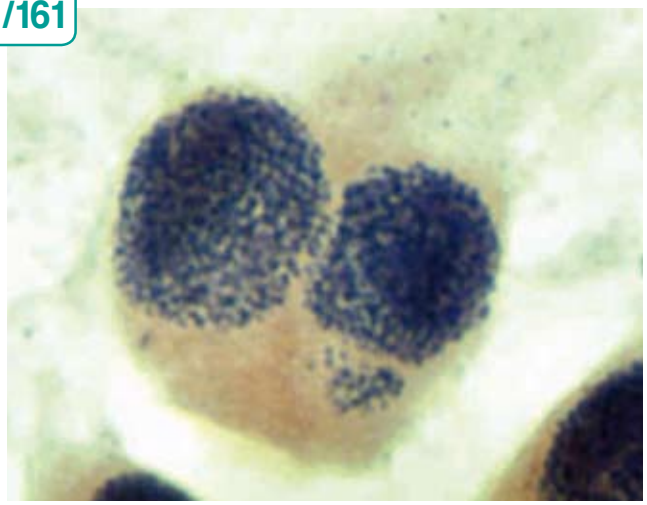


8/161



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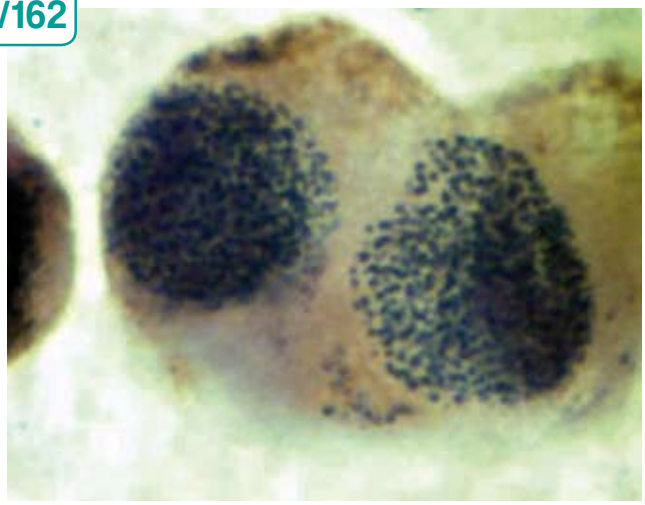

➔ **New nuclei multipolar divisions: many new nuclei are generated and released from cells, and they contained Gram-positive cocci** (×2000 Gram staining) (Figures **8/163** to **8/186**)

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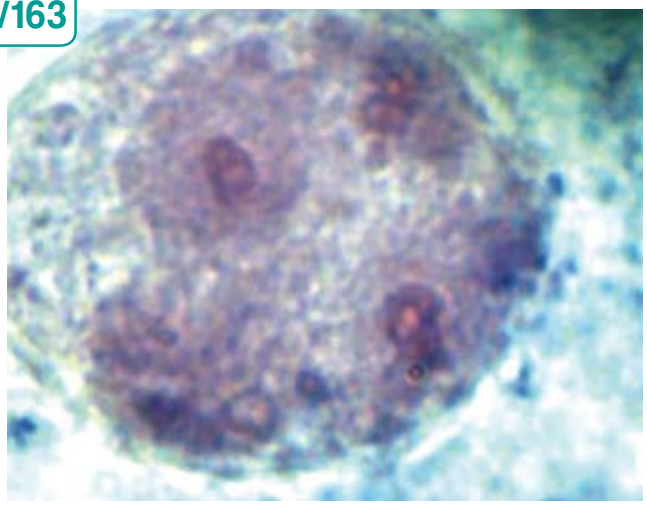
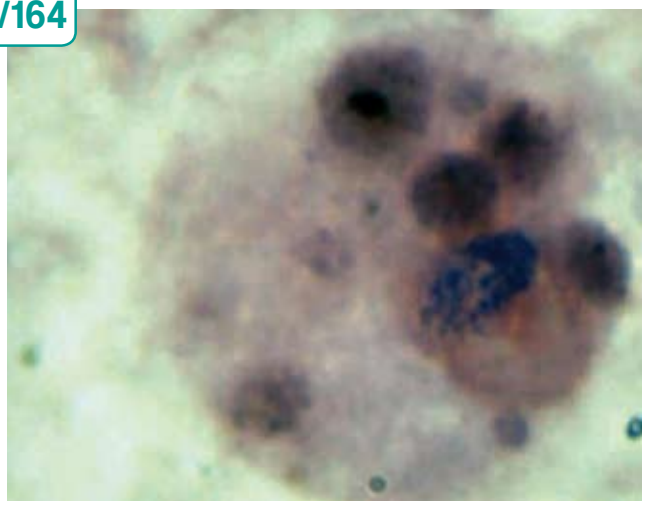
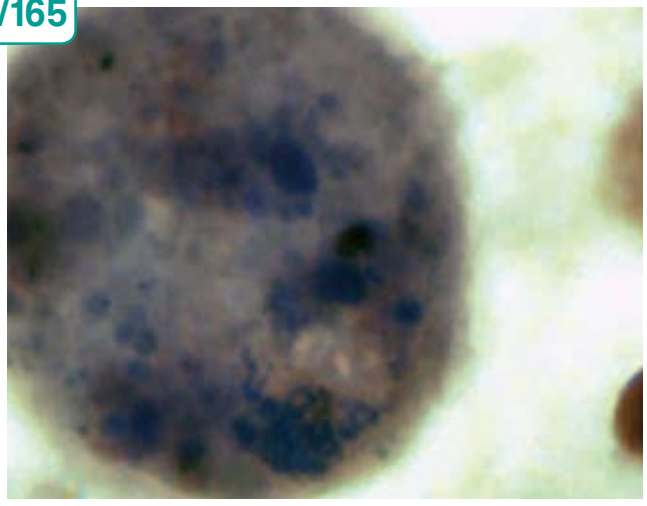


8/164



8/165



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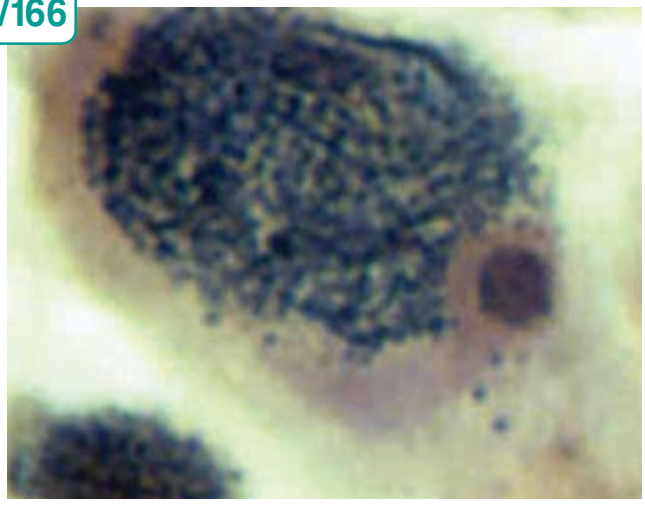
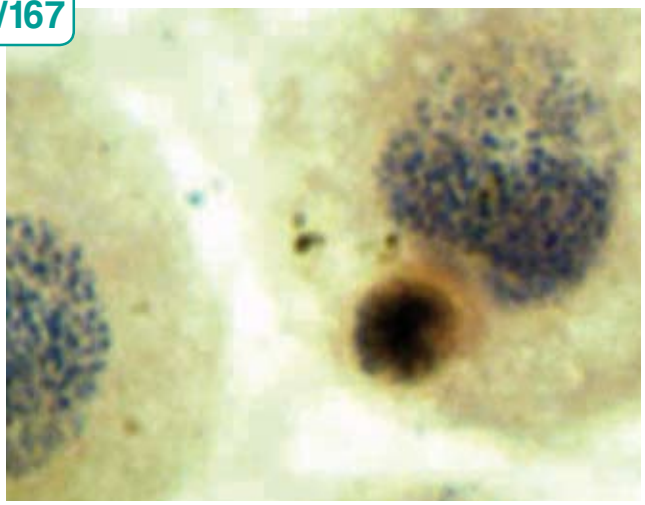
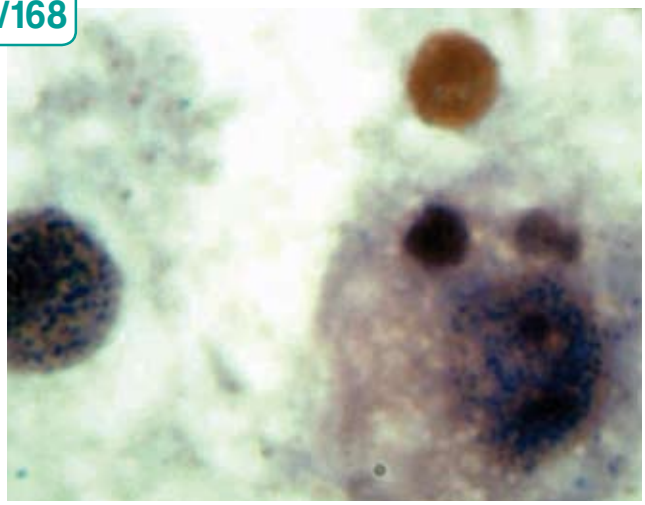


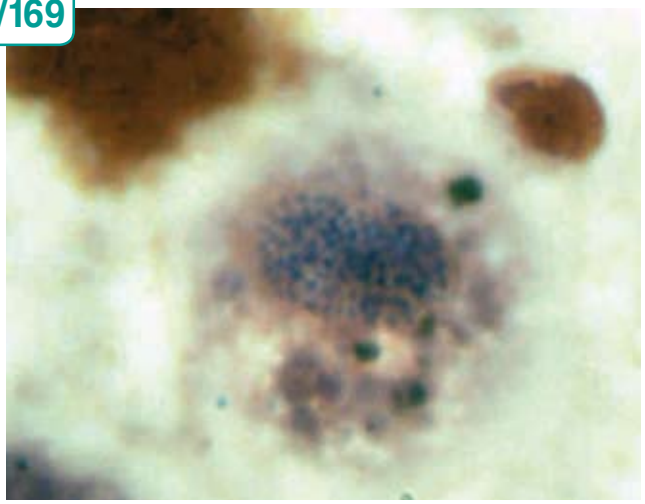
8/167



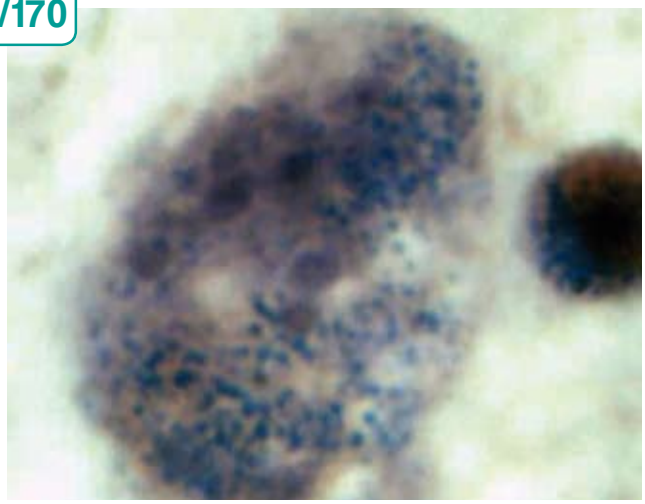
8/168



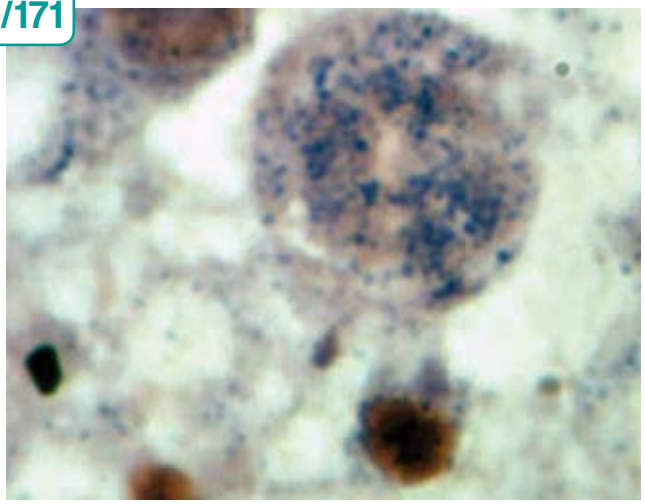
8/169



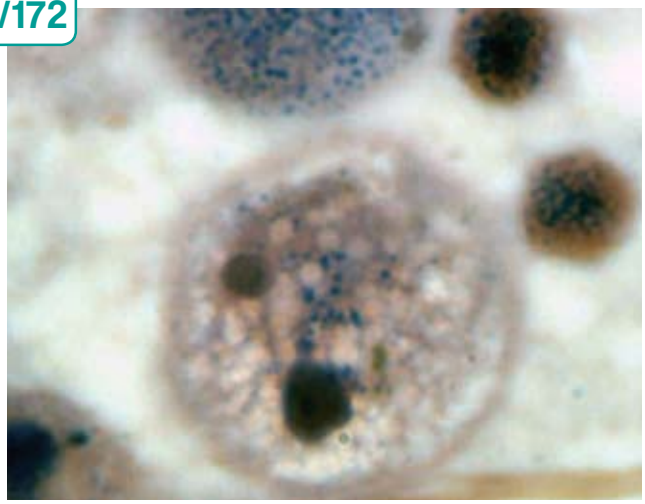
8/170



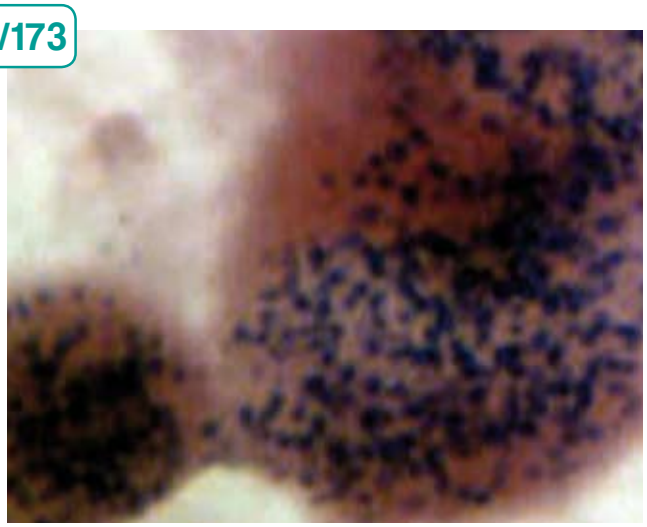
8/171



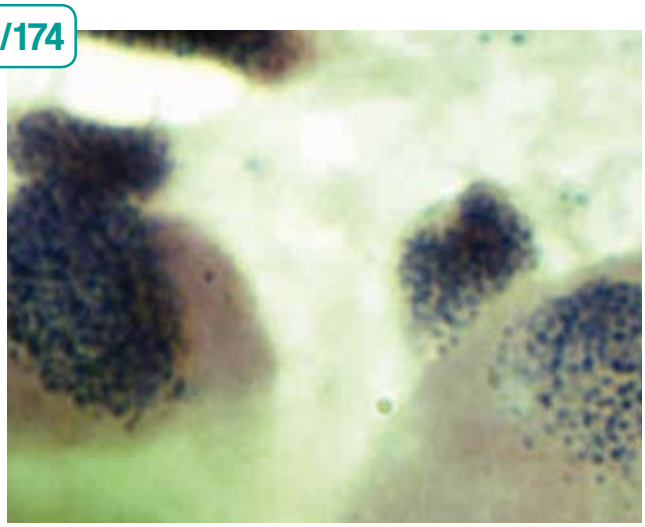
8/172



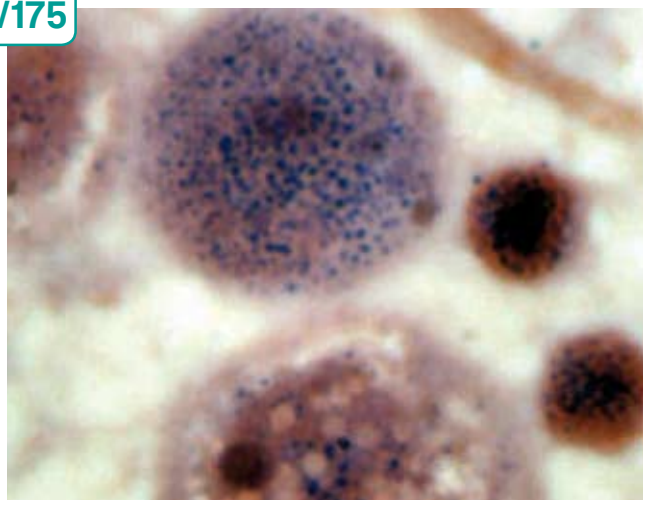
8/173



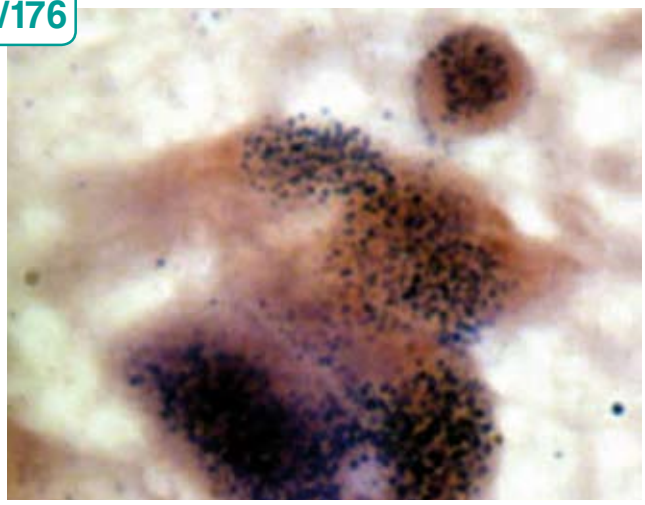
8/174



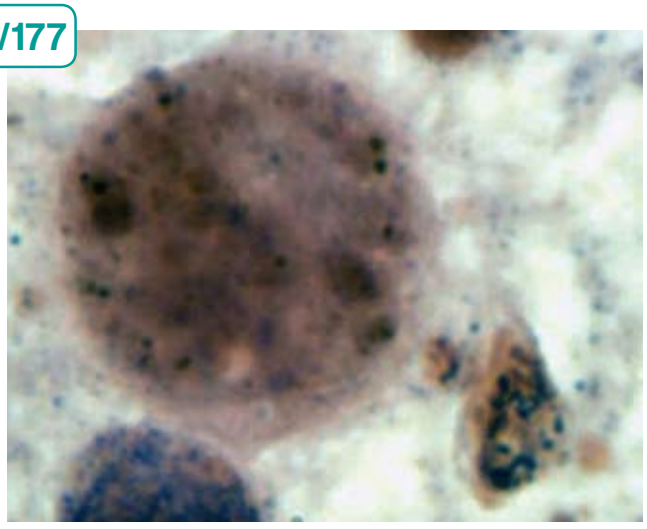
8/175



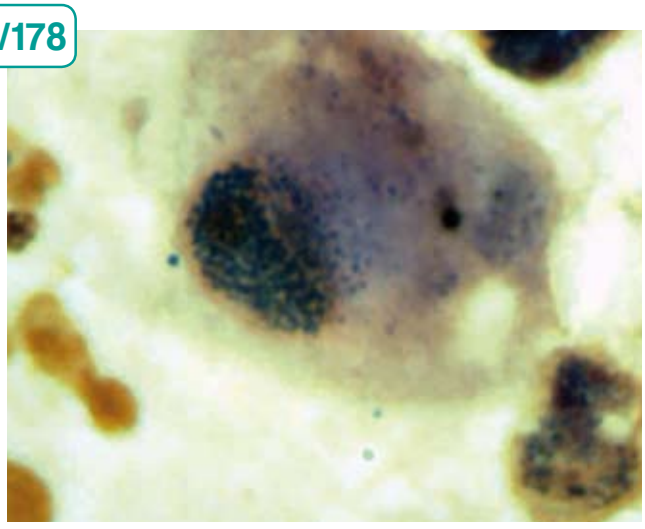
8/176



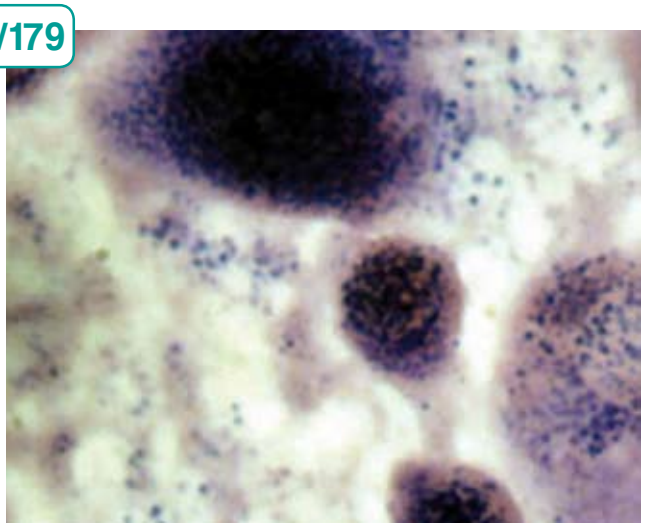
8/177



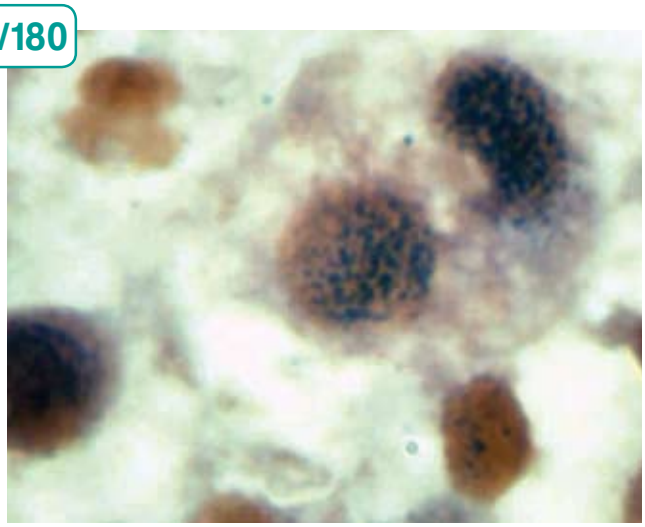
8/178



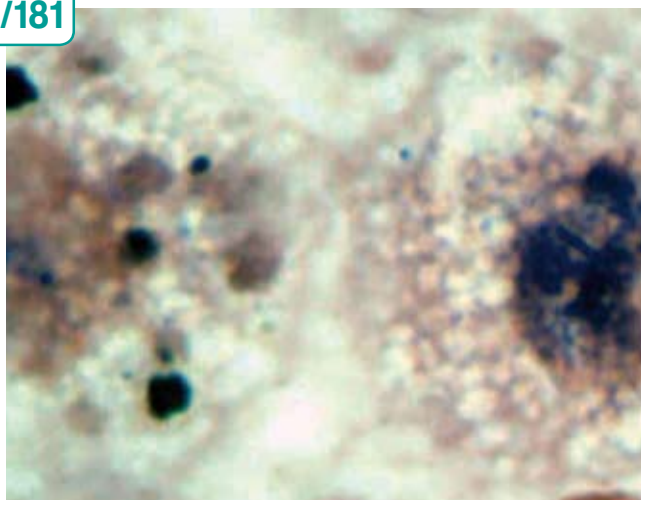
8/179



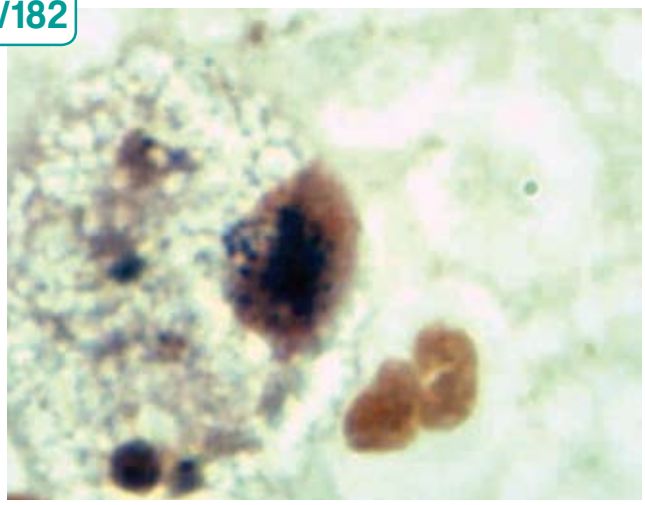
8/180



8/181



8/182



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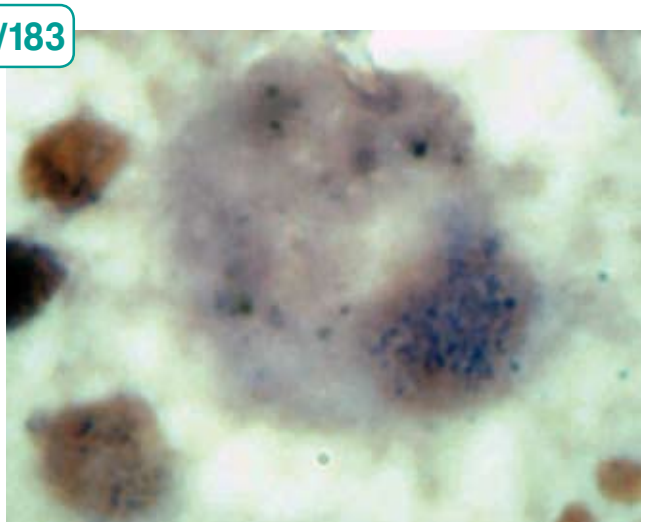

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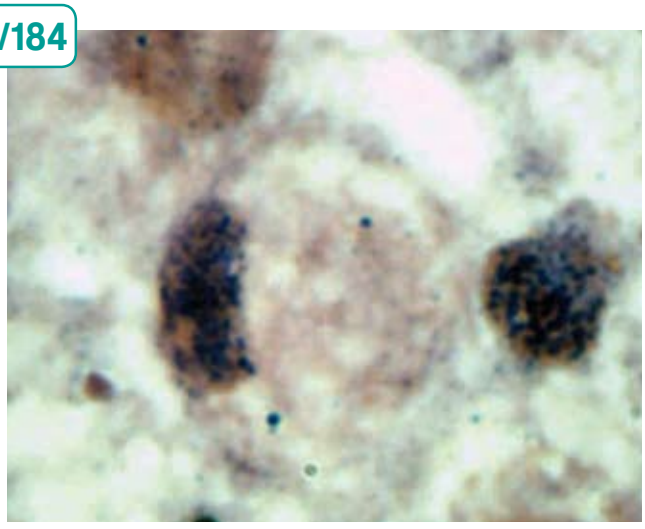
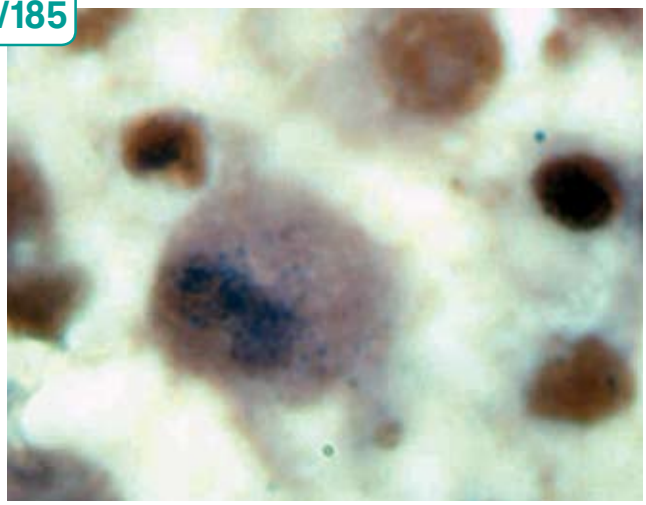


8/185



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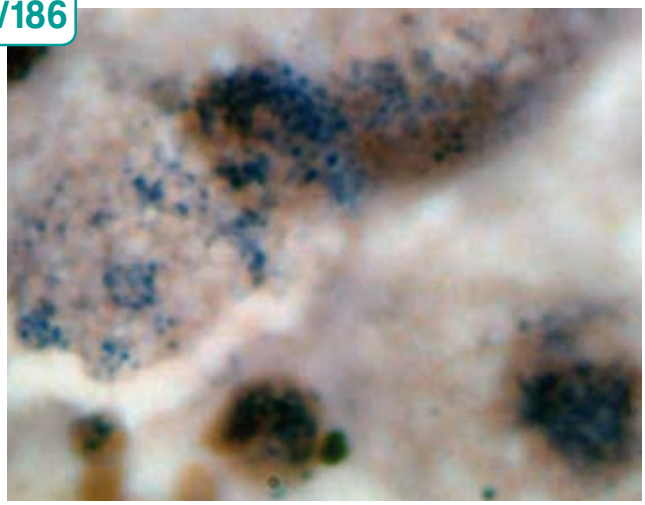

→ **New nuclei leave cells by membrane disruption in poor viability cells** (Syto 9 plus propidium iodide staining) (Figures **8/187** and **8/188**)

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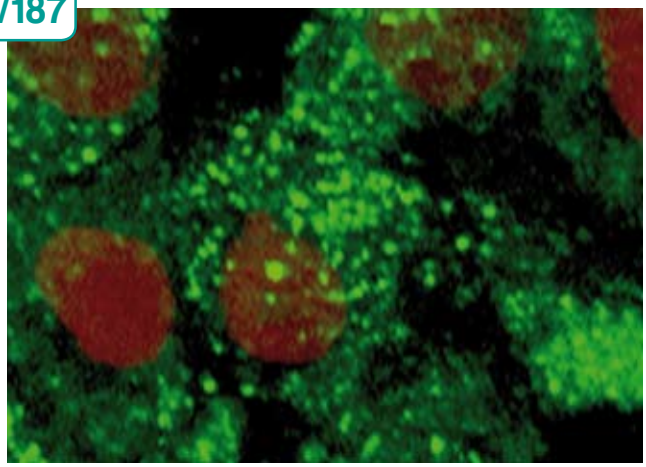
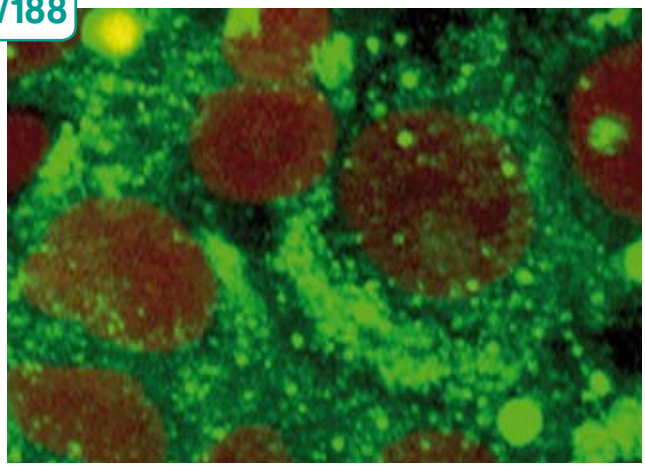
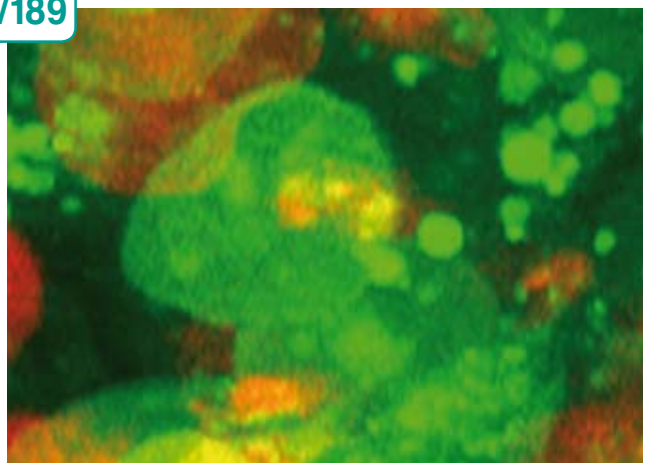


8/188



→ **New nuclei leave cell trough "holes" in cellular membrane** (Syto 9 plus Propidium iodide) (Figures **8/189** and **8/190**)

8/189



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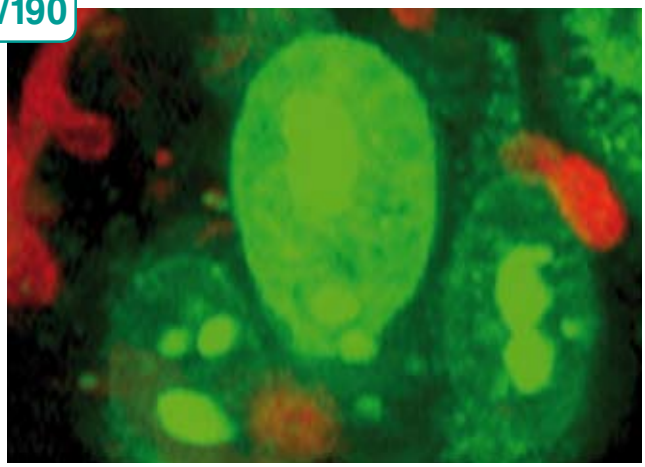
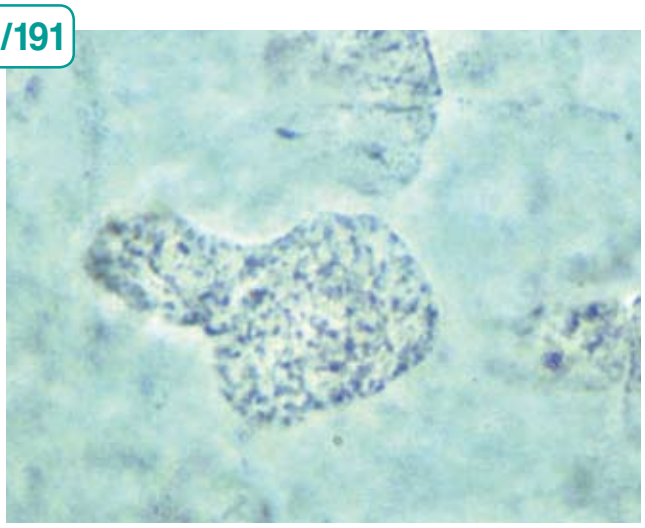
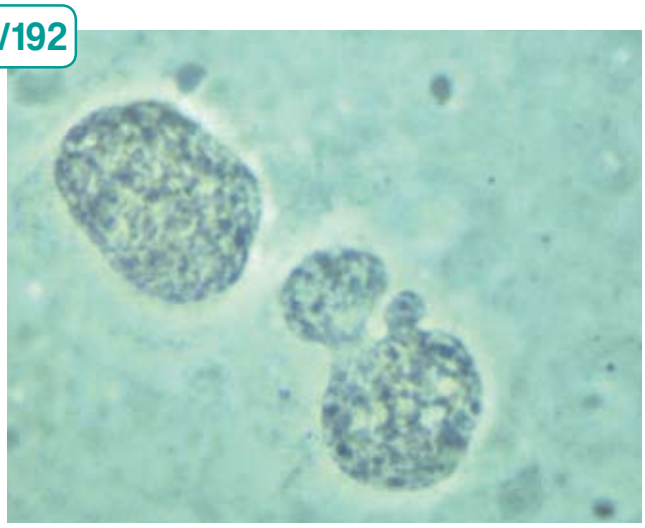


→ **New nuclei leave cell with part of the cytoplasm** (Lacto-orcein staining) (Figures **8/191** to **8/197**)

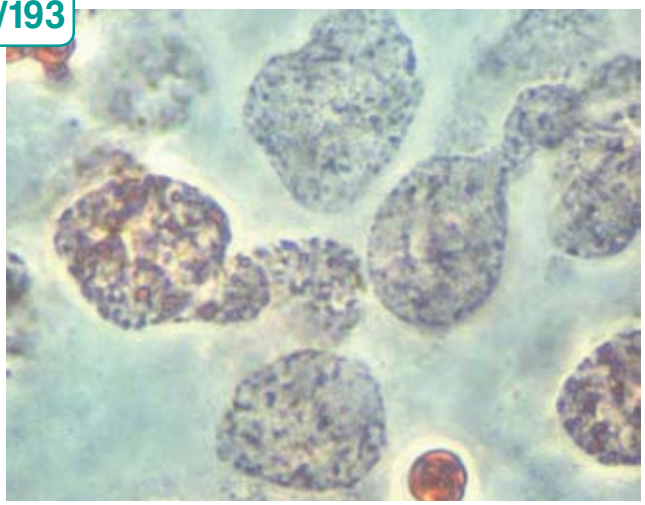
8/191



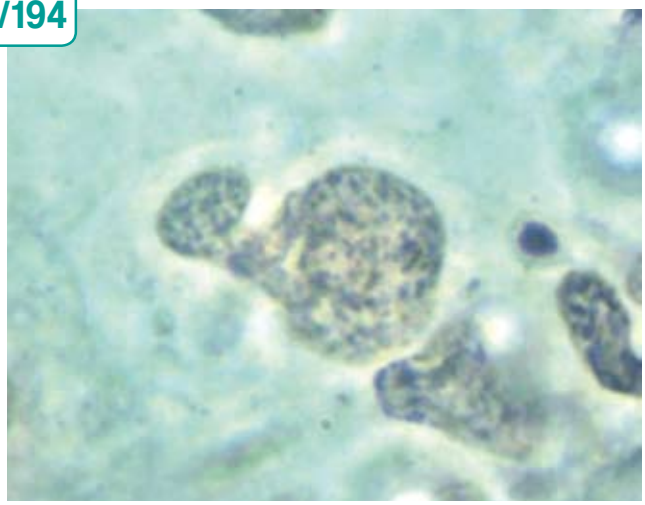
8/192



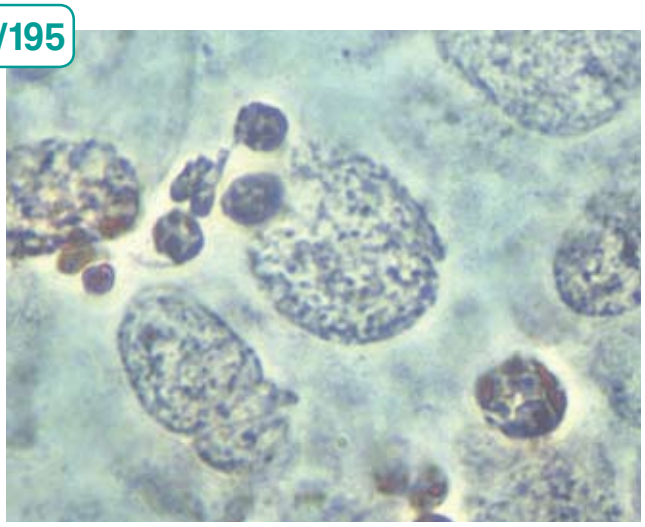
8/193



8/194



8/195



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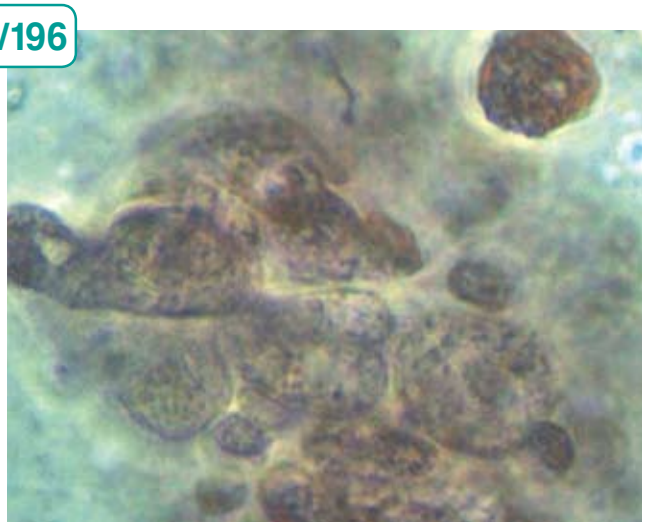
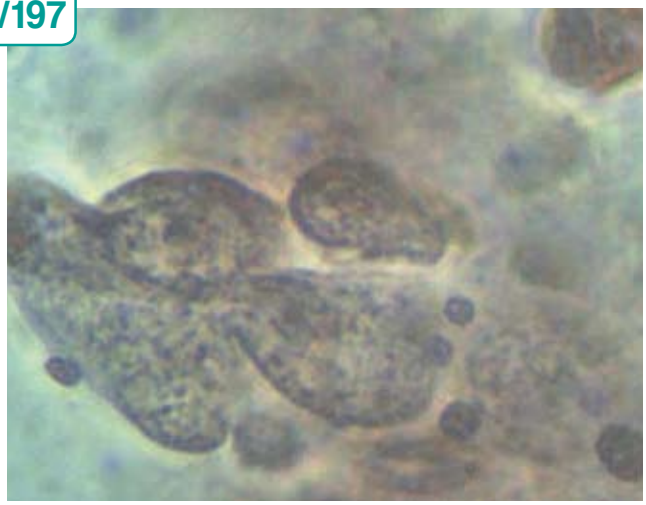
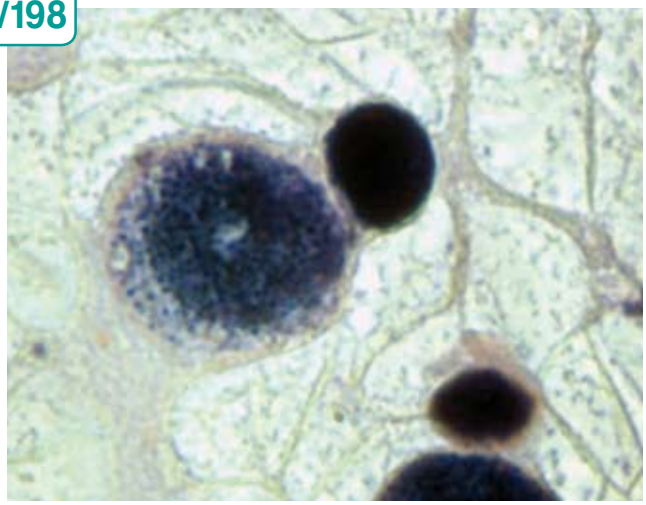


8/197

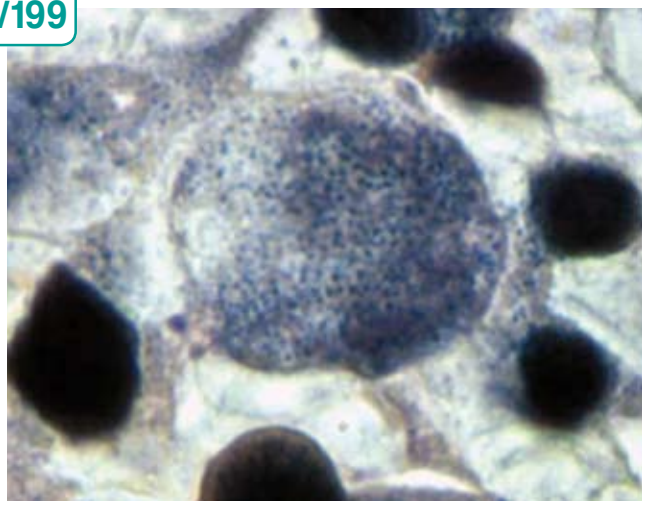


➔ **New nuclei leave cell with part of the cytoplasm** (×2000 Gram staining) (Figures **8/198** to **8/202**)

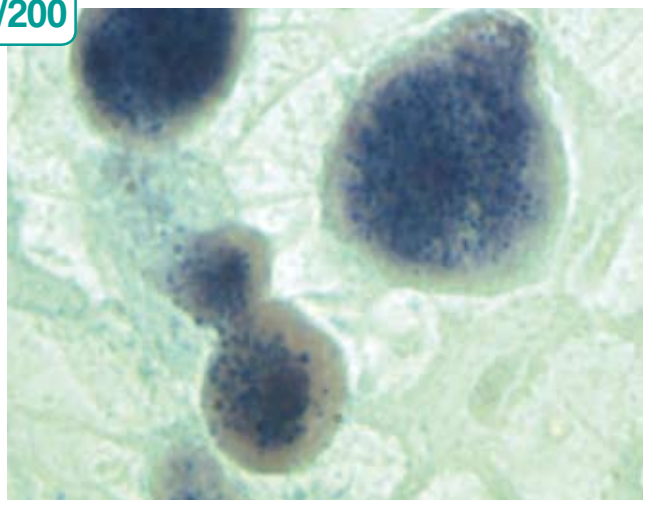
8/198



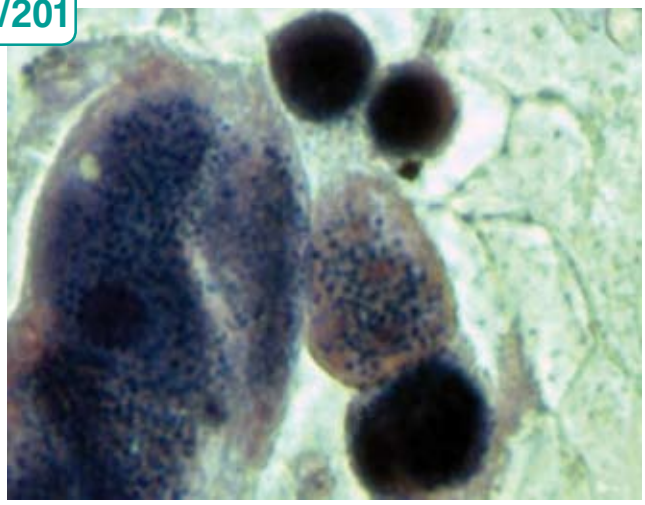
8/199



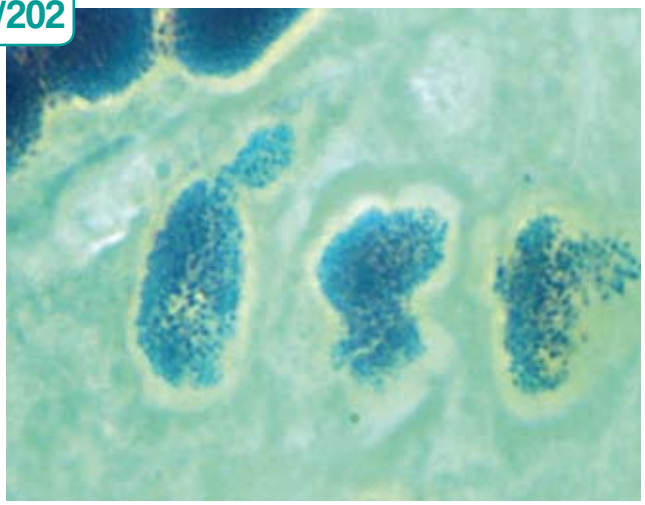
8/200



8/201



8/202



→ **Empty cells, after releasing new nuclei and extranuclear bacteria** (Syto 9 plus propidium iodide) (Figures **8/203** to **8/205**)

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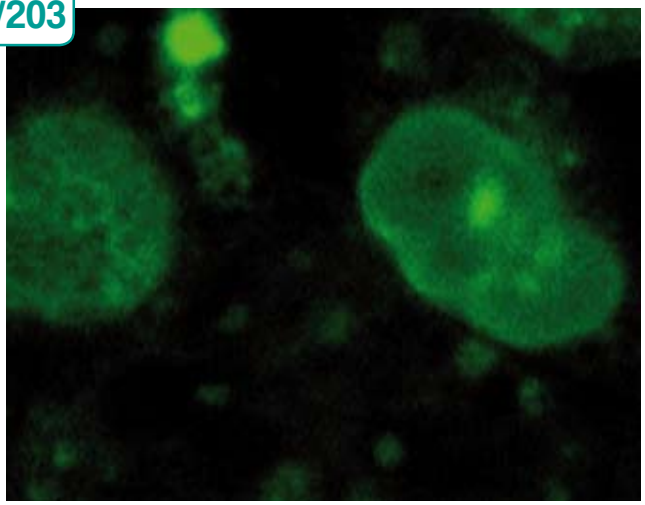

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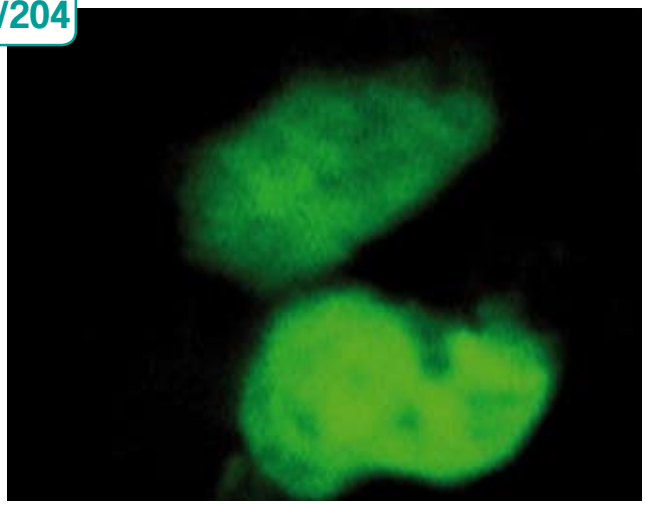
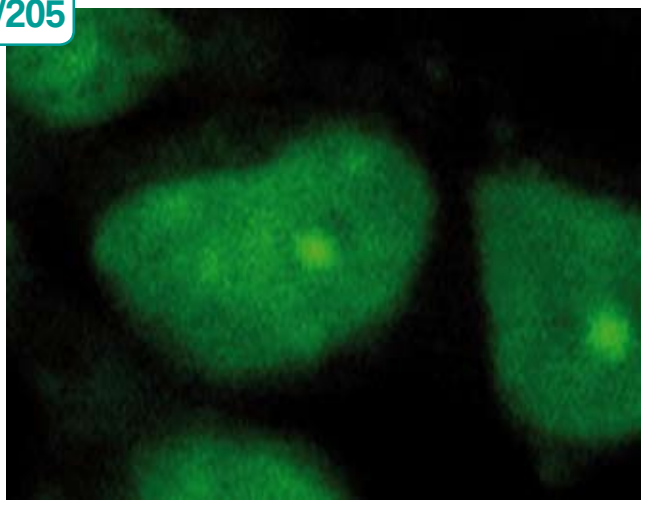


8/205



➔ **Empty cell** (×2000 Gram staining) (Figure **8/206**)

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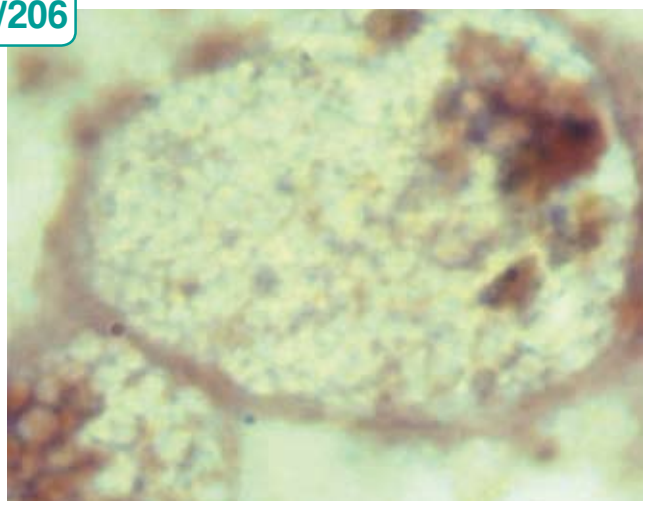
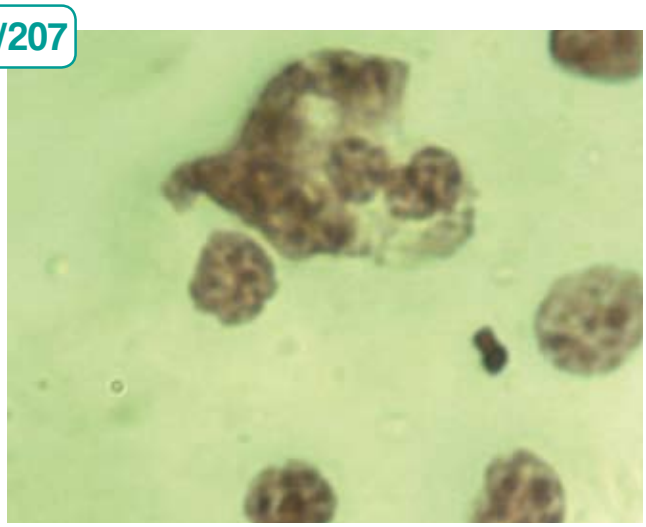


➔ **Empty cell** (×2000 Lacto-orcein staining) (Figure **8/207**)

8/207



→ **Some dead nuclei (red) contain cavities with living bacteria (green)** (Syto 9 plus propidium iodide) (Figures **8/208** to **8/216**)

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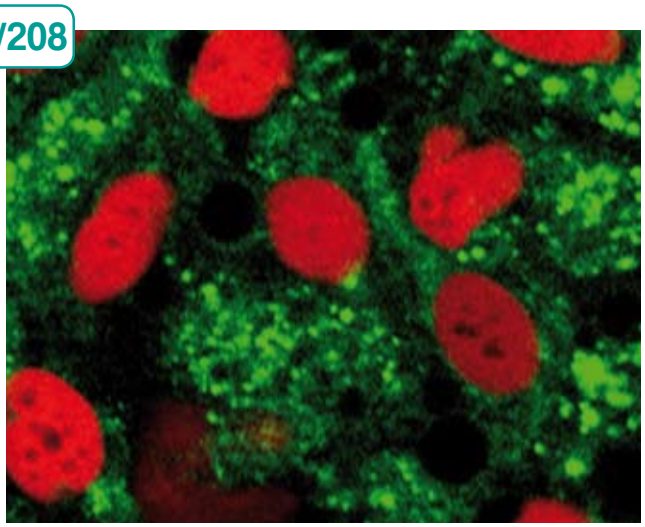

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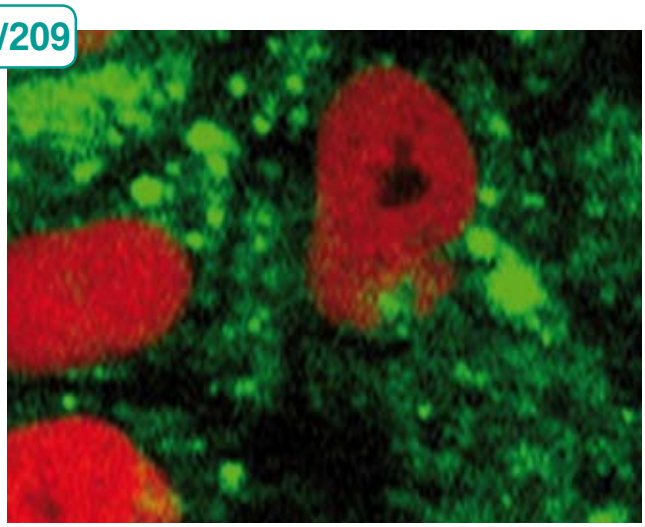
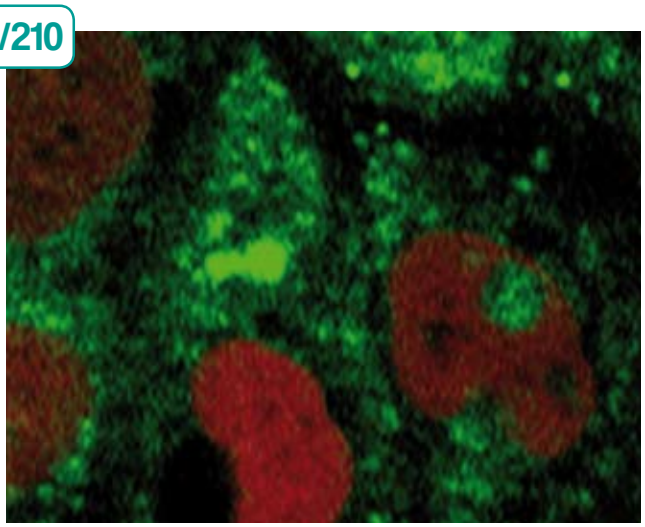
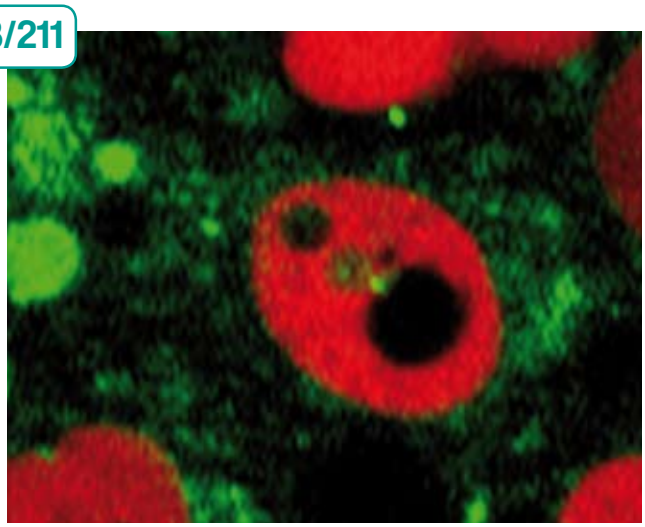


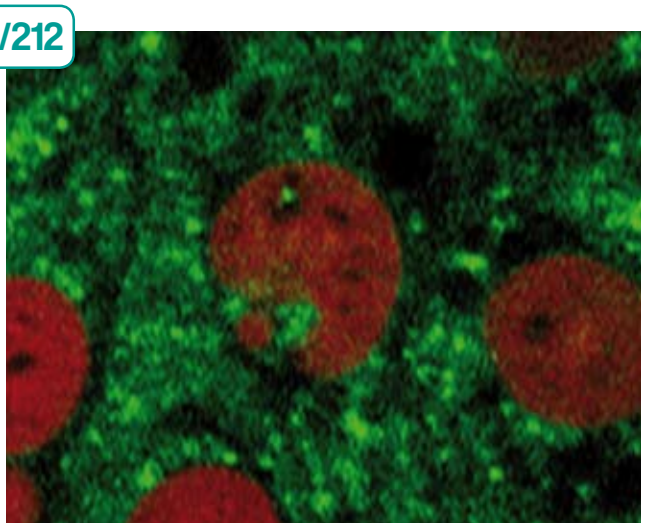
8/210



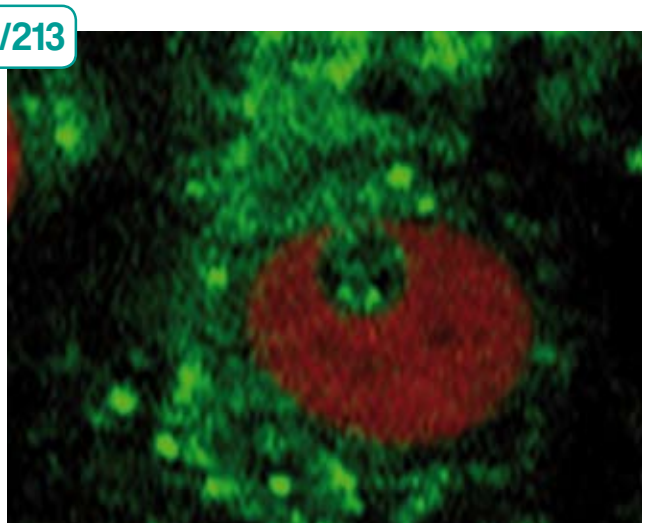
8/211



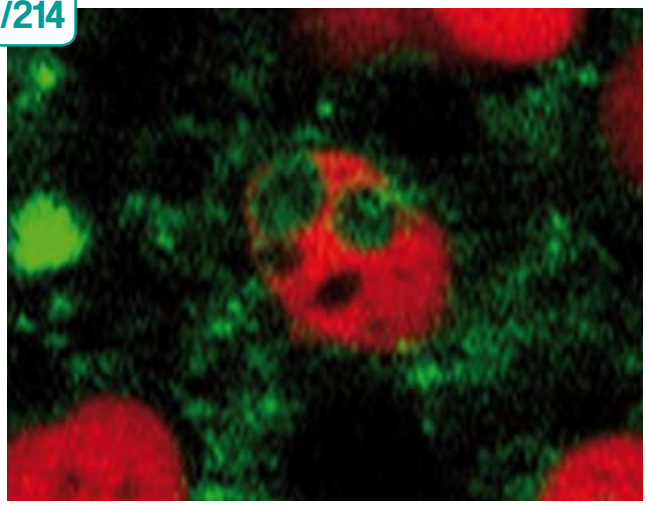
8/212



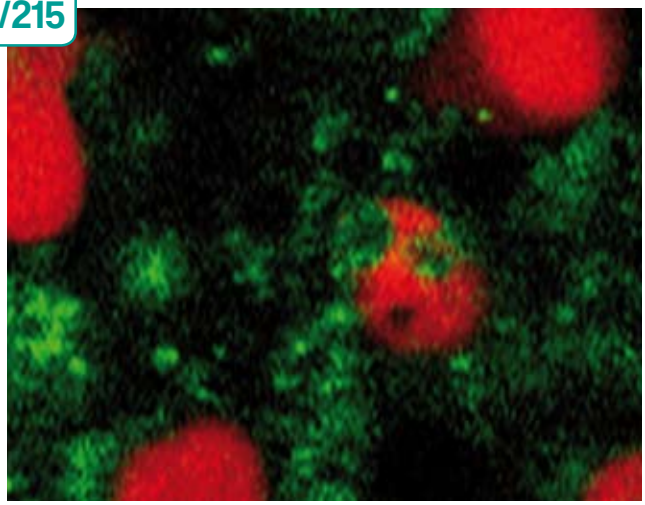
8/213



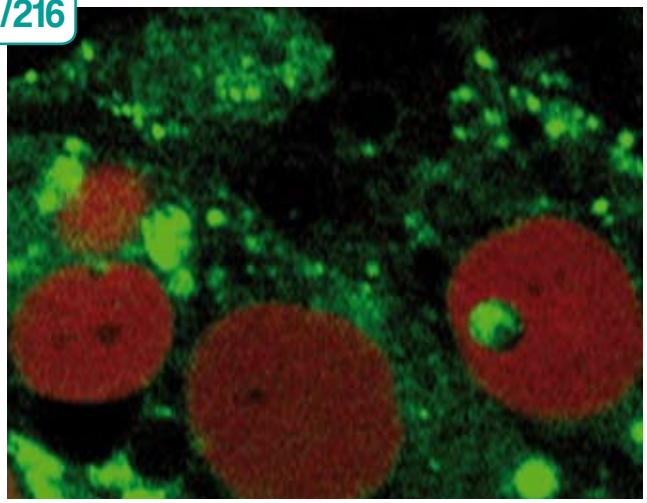
8/214



8/215

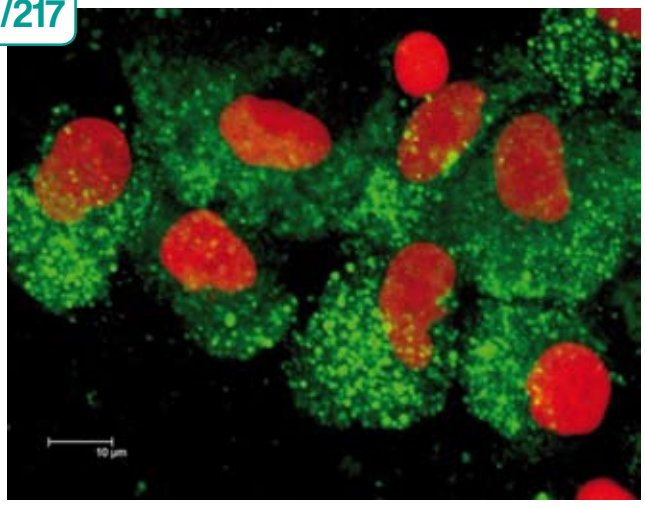


8/216



→ **Crowded living bacteria (green) observed around dead cell (red)** (Syto 9 plus Propidium iodide: these cells have been stained within 6 min. of surgical procedure) (Figures **8/217** to **8/219**)

8/217



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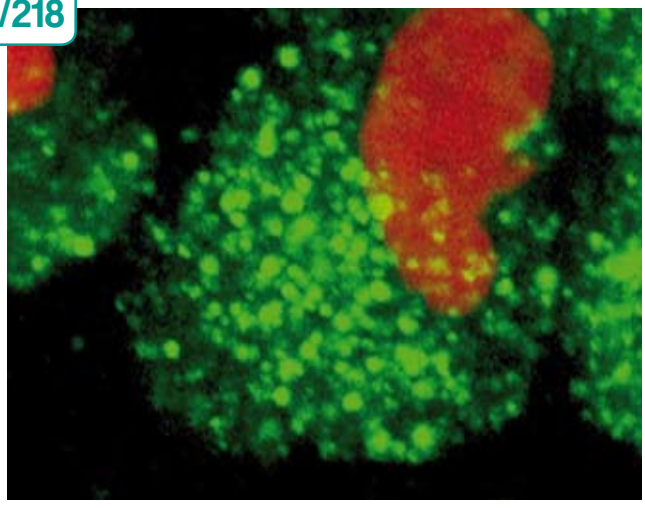
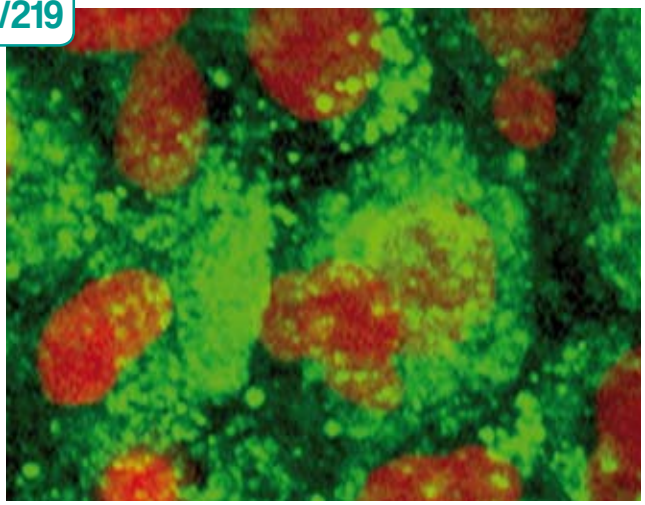
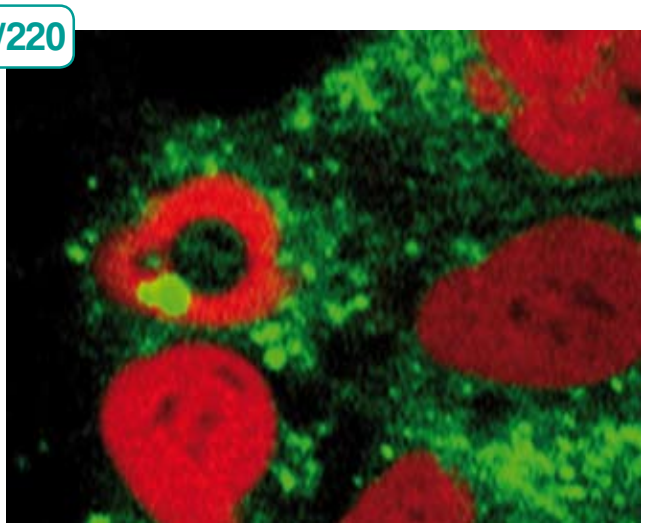


8/219

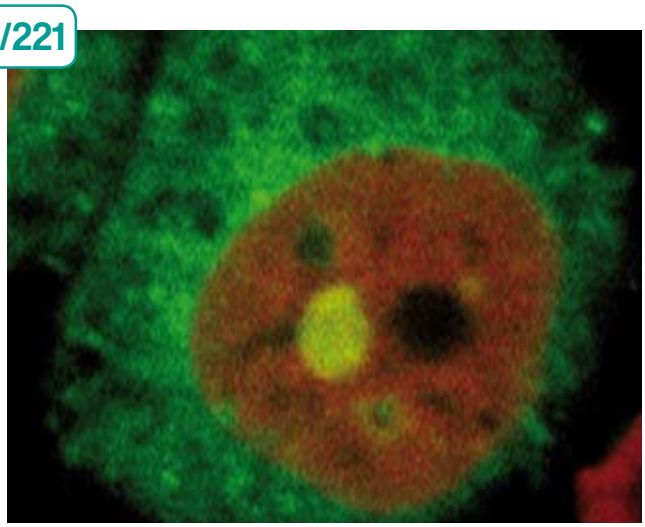


→ **Dead cells (red) containing new living nuclei(green) inside nucleus or cytoplam** (Syto 9 plus propidium iodide) (Figures **8/220** to **8/223**)

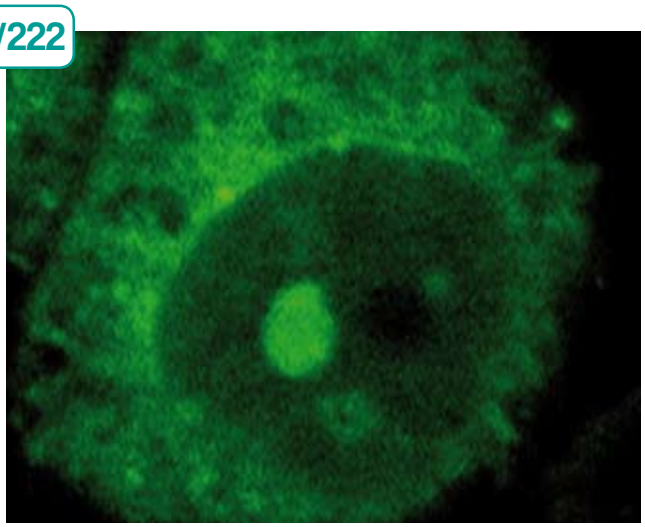
8/220



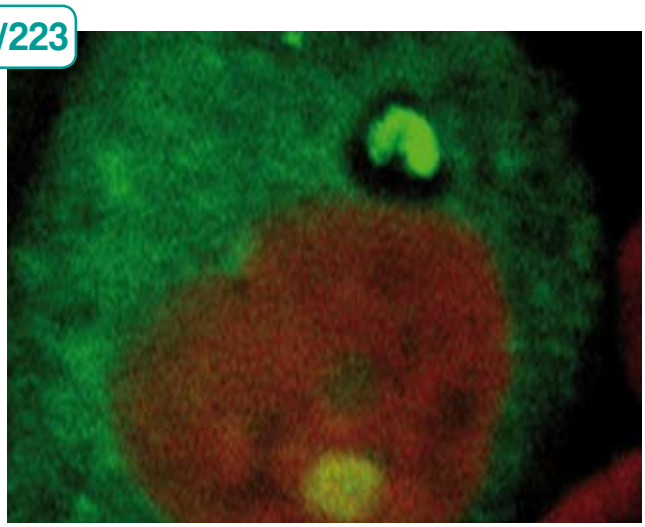
8/221



8/222

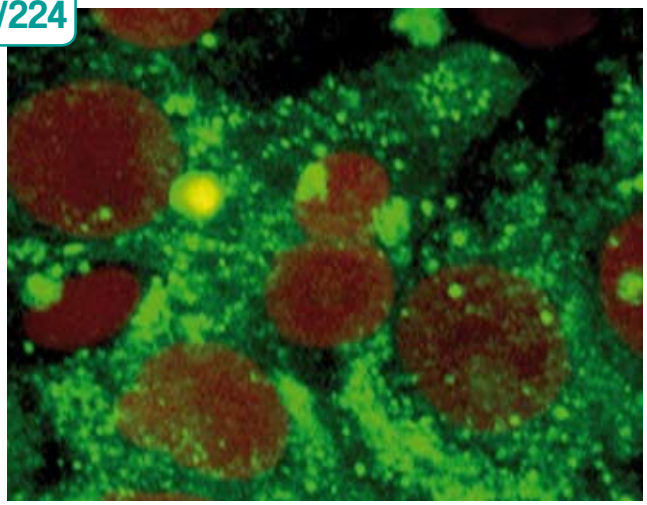


8/223

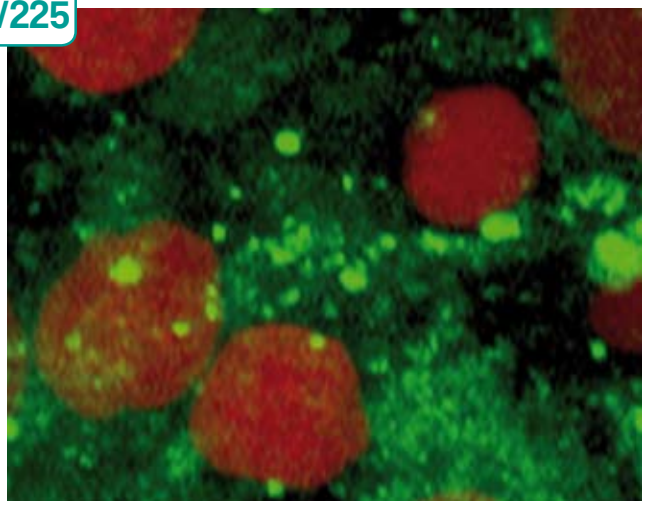


→ **Risk of spontaneous or induced tumoral necrobiosis.** Damaged cell membranes disappear, leaving new nuclei and extranuclear bacteria free and widespread (Syto 9 plus Propidium iodide) (Figures **8/224** to **8/226**)

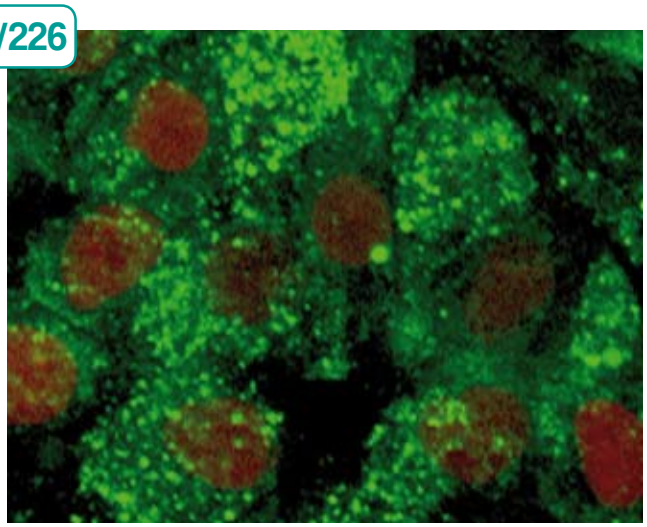
8/224



8/225



8/226



CHAPTER 9

BACTERIAL CULTURES FROM NON-NEOPLASIC LUNG AND PULMONARY CANCER

1. Introduction

The presence of intracellular bacteria inside tumors needs to be confirmed by their growth in culture media and ulterior identification. Gram-positive cocci can be found not only in living but also in dead tumoral cells, and therefore, their growth in culture media should be possible.

2. Materials and methods

For 1 year, samples of neoplasms and neighboring non-tumoral lung tissue were prospectively inoculated directly onto culture media. These samples came from specimens 35–57 (all inclusive; Table 9/1). Samples were processed within 60 min of surgery procedure with an autonomous laminated flow cabin (Telstar bio II A/P). Samples were cut in small fragments, on sterile conditions, and inoculated with thioglycollate directly into aerobic and anaerobic haemoculture media (BD Bacteplus aerobic/anaerobic) and on blood agar, chocolate agar, and Levine Petri dishes and incubated at 37°C. The plates were inspected after 24 h, 48 h, and 72 h, and suspected colonies underwent further characterization.[1] Bacteria isolates were identified and characterized by the use of conventional microbiological methods.

3. Results

23 samples came from non-tumoral lung tissue, 22 from lung cancer tissue, and one sample from an inflammatory pseudotumor. The histopathological types of these samples corresponded to 9 squamous-cell carcinomas, 9 adenocarcinomas, 2 large-cell neuroendocrine carcinomas, 1 metastatic cancer and 1 pleomorphic sarcoma (Table 9/2).

By error, the bacteriological samples for the specimen number 41 were not sent to the laboratory. Excluding the inflammatory pseudotumor from which *Klebsiella pneumoniae* were grown, results were positive for bacteria in 10 of the 21 tumor samples and 7 of the 22 benign samples, without any statistically significant differences. Bacteria isolated from malignancies were of a wide variety of Gram-positive and Gram-negative rods and cocci. The presence of Gram-positive cocci did not differ significantly between cultures coming from tumor or benign samples (Table 9/1).

4. Discussion

First, despite the reported high number of living bacteria inside tumor cells, and the careful process and technology used in this study, only half of tumor samples grew any microbes. This is a similar proportion to that of normal lung samples. Secondly, in contrast to the single morphology (Gram-positive cocci) noted with lacto-orcein, Gram, and fluorochrome staining, the bacterial cultures showed a variety of microbes, suggesting that the bacteriological cultures had no relation with the possible endonuclear symbionts described earlier. These bacteriological results could be explained by contamination that could come from sample handling, despite the quick processing and sterility, or by the presence of bacteria in the tissue dissected itself. Moreover, normal lung parenchyma and airways are known to contain bacteria, whose presence increases when there are pathologies. If the presence of bacteria were due to contamination while handling the samples, both tumor and non-tumoral lung samples would contain the same bacteria, because both are exposed equally to the same potential contamination sources during their manipulation.

[1] Brewer Ns, Weed La. *Diagnostic tissue microbiologic method.* Human Pathol 1976; 7: 141–47.

Germs coincided in both tumor and neighboring normal lung samples only in case numbers 36 and 52. Thus, because in nine cases germs differed from tumor and normal lung samples, the hypothesis of contamination is less likely. Despite the massive and generalized presence of intracellular cocci in tumor tissue, these inconclusive results were expected. Most known endosymbionts do not grow outside their host cells. Because of their defective metabolic pathways, they depend on the products metabolized by their host cell for survival and reproduction. Only occasionally the growth of symbionts outside their host cell has been described, and these results are doubtful.[2] These points will be further discussed in Chapters 10 to 12. In summary, classical bacteriological cultures are of no use for isolation and identification of these intracellular living bacteria, found in neoplastic cells.

[2] Horn M, Wagner M. *Bacterial endosymbionts of free-living amoebas.* J. Eukaryot Microbiol 2004; 51: 509–14.

Table 9/1. BACTERIAL ISOLATED FROM MALIGNANT AND BENIGN SAMPLES.

Nº	AGE (years)	GENDER	TYPE OF TUMOR	BACTERIAL CULTURES		TYPE OF LUNG RESECTION
				CANCER	NON-TUMORAL LUNG	
35	66	MALE	LARGE-CELL NEURENDOCRINE CANCER	STAPHYLOCOCCUS EPIDERMIDIS STREPTOCOCCUS SP NEISSERIA SP	NON BACTERIA GROWTH	LOBECTOMY
36	73	MALE	SQUAMOUS CELL	STAPHYLOCOCCUS AUREUS STREPTOCOCCUS SP CORYNEBACTERIUM SP	STREPTOCOCCUS SP CORYNEBACTERIUM SP	LOBECTOMY
37	74	MALE	SQUAMOUS CELL	SERRATIA LIQUEFACIENS	STAPHYLOCOCCUS EPIDERMIDIS	NEUMONECTOMY
38	58	MALE	SQUAMOUS CELL	STREPTOCOCCUS SP	NON BACTERIA GROWTH	ATYPICAL RESECTION
39	49	FEMALE	LARGE-CELL NEURENDOCRINE CANCER	NEISSERIA SP	NON BACTERIA GROWTH	LOBECTOMY
40	71	FEMALE	ADENOCARCINOMA	NON BACTERIA GROWTH	STAPHYLOCOCCUS EPIDERMIDIS	NEUMONECTOMY
41	67	MALE	ADENOCARCINOMA	SPECIMEN NOT SENDING TO LABORATORY		
42	49	MALE	SQUAMOUS CELL	NON BACTERIA GROWTH	NON BACTERIA GROWTH	NEUMONECTOMY
43	55	MALE	ADENOCARCINOMA	NON BACTERIA GROWTH	STAPHYLOCOCCUS EPIDERMIDIS	LOBECTOMY
44	58	MALE	INFLAMMATORY PSEUDOTUMOR	NON BACTERIA GROWTH	KLEBSIELLA PNEUMONIAE	LOBECTOMY
45	74	MALE	PLEOMORPHIC SARCOMA	NON BACTERIA GROWTH	ENTEROCOCCUS FAECALIS	LOBECTOMY
46	59	MALE	SQUAMOUS CELL	STREPTOCOCCUS SP	NON BACTERIA GROWTH	LOBECTOMY
47	74	MALE	SQUAMOUS CELL	BACILLUS SP	NON BACTERIA GROWTH	LOBECTOMY
48	58	FEMALE	METASTATIC CANCER	STREPTOCOCCUS SP	NON BACTERIA GROWTH	LOBECTOMY
49	70	MALE	ADENOCARCINOMA	NON BACTERIA GROWTH	NON BACTERIA GROWTH	NEUMONECTOMY
50	63	MALE	SQUAMOUS CELL	NON BACTERIA GROWTH	NON BACTERIA GROWTH	LOBECTOMY
51	55	MALE	ADENOCARCINOMA	NON BACTERIA GROWTH	NON BACTERIA GROWTH	LOBECTOMY
52	76	MALE	ADENOCARCINOMA	BACILLUS SP	BACILLUS SP	LOBECTOMY
53	78	MALE	ADENOCARCINOMA	NON BACTERIA GROWTH	NON BACTERIA GROWTH	ATYPICAL RESECTION
54	81	MALE	SQUAMOUS CELL	NON BACTERIA GROWTH	NON BACTERIA GROWTH	ATYPICAL RESECTION
55	58	MALE	ADENOCARCINOMA	NON BACTERIA GROWTH	NON BACTERIA GROWTH	LOBECTOMY
56	71	MALE	SQUAMOUS CELL	STAPHYLOCOCCUS AUREUS	NON BACTERIA GROWTH	NEUMONECTOMY
57	52	MALE	ADENOCARCINOMA	NON BACTERIA GROWTH	NON BACTERIA GROWTH	LOBECTOMY

Table 9/2. SOCIODEMOGRAPHICS AND TUMOR CHARACTERISTICS. BACTERIAL CULTURES FROM NON NEOPLASIC LUNG AND PULMONARY CANCER.

AGE (years)	MEAN ± 1SD. (MAXIMUM, MINIMUM)	64.8 ± 9.6 (49-81)		
GENDER	FEMALE	3 (20.0%)		
	MALE	20 (87.0%)		
TYPE OF TUMOR	ADENOCARCINOMA	9 (39.1%)		
	SQUAMOUS- CELL CANCER	9 (39.1%)		
	METASTATIC CANCER	1 (4.3%)		
	LARGE-CELL NEURENDOCRINE CANCER	2 (8.7%)		
	PLEOMORPHIC SARCOMA	1 (4.3%)		
	INFLAMMATORY PSEUDOTUMOR	1 (4.3%)		
TNM DESCRIPTOR		T	N	M
	0		16 (69.6%)	4 (17.4%)
	1	3 (13.0%)	3 (13.0%)	1 (4.3%)
	2	15 (65.2%)	3 (13.0%)	
	3	4 (17.4%)		
	4	1 (4.3%)		
	X		1 (4.3%)	18 (78.3%)
TYPE OF LUNG RESECTION	ATYPICAL RESECTION	3 (13.0%)		
	LOBECTOMY	14 (60.9%)		
	NEUMONECTOMY	6 (26.1%)		

CHAPTER

10 IDENTIFICATION BY MALDI-TOF MASS SPECTROMETRY AND 16S rRNA GENE SEQUENCING

1. Introduction

Because the Gram-positive cocci found inside neoplasic cells could not be grown in the bacterial culture media, their identification had to be done by other methods. Matrix-Assisted Laser Desorption Ionization (MALDI) Time Of Flight (TOF) Mass Spectrometry (MS) has been developed for protein analysis during the past decades. The principle of MS is to produce, separate, and detect gas-phase ions from proteins trapped in a band from a gel. When identification failed with MALDI-TOF, then the samples were analyzed by sequencing 16 ribosomal RNA-based identification systems for bacteria.[1]

2. Materials and methods

20 samples from 10 consecutive tumors and 9 from non-neoplasic lungs obtained from surgical procedures were immediately cut in small fragments on aseptic conditions, and placed in the middle of two sterile glass sheets of 25 × 5 cm. The samples were crushed with a clamp for 5 min. After that, the samples were collected and centrifuged by 1000×g for 1 min. The pellets, containing bulky material, were discarded and the supernatant was centrifuged by 5000×g during 12 min. The new pellet was suspended in 300 µL of distilled RNAse free water (Sigma-Aldrich, St Louis, Mo) and vortexed. After that, 900 µL of 100% ethanol (Sigma- Aldrich) were added, vortexed, and centrifuged at top speed (5000×g) for 12 min. Supernatant was discarded, and the pellet was again centrifuged at top speed. Residual supernatant was removed and the pellet was dried at room temperature. 50 µL of 70% formic acid (Fluka, Sigma-Aldrich, St Louis, Mo) were added to the pellet and mixed by pipeting. Subsequently, 50 µL of acetronile (Fluka) were added and thoroughly mixed, followed by centrifugation at top speed for 12 min. 1 µL of supernatant was placed on the plate and allowed to dry at room temperature before the addition of 1 µL of matrix solution (saturated solution of alpha-cyano-4 hidroxycinnamic acid in 50% acetronile and 2.5% trifluoroacetic acid). The matrix was crystallized by air drying at room temperature. Measures were performed with a Microflex mass spectrometer (Brucker Daltonik, Bremen, Germany) using Flex Control Sofware (version 3.0). Spectra were recorded in the positive linear mode (laser frequency 20 Hz; ion source 1 voltage 20 kv; spectrum 240 shots in 40 shot steps from different positions of the target). Spot were collected and analyzed by standard pattern-matching with Byo Typer database. Results of the pattern-matching process were scored ranging from 0 to 3. Scores below 1.7 were considered not to have generated a reliable identification.[2][3][4]

In the case of non-identification, the results of sequencing the 16S rRNA gene (or 16S DNA) were the final and definitive means of identification. A crude DNA extract was prepared as follows: bacterial genomic DNA was extracted from cells with Pre Man extraction reagent. A small amount of the sample was suspended in 1 ml of sterile saline and incubated at 95°C for 10 min. Lysed suspensions were then centrifuged at 5000×g for 12 min. 1 µL of supernatant was amplified by PCR. The PCR reaction mixture was as follows: 1×PCR buffer, 15 mM $MgCl_2$; 0.1 mMd NTPs, 1 U Taq polymerase (all Invitrogen); 0.4 µM of each of the following primers: 16 Fa, 16 Fb, 16 Sr, and sterile

[1] Weisbourg WG, Barns SM, Pelletier DA, Lane DJ. *16 S ribosomal DNA amplification for phylogenetic study.* J Bacteriol 1991; 173: 697-703.
[2] Eigner U, Holfelder M, Oberdorfer K, Betz-Wild U, Bertsch D, Fahr AM. *Performance of matrix- assisted desorption ionization- time -of-flight mass spectrometry system for the identification of bacterial isolates in the clinical routine laboratory.* Clin Lab 2009; 55: 289-96.
[3] Bizzini A, Greub G. *Matrix- assisted laser desorption ionization time- of- flight mass spectrometry, a revolution in clinical microbial identification.* Clin Microbiol Infect 2010;16: 1614-9.
[4] Alatoom AA, Cunningham SA, Ihde SM, Mandrekar J, Patel R. *Comparison of direct colony method versus extraction method for identification of Gram- positive cocci by use Brucker Biotyper Matrix- assisted laser desorption ionization - time of flight mass spectrometry.* J Clin Microbiol 2011; 49: 2868-73.

UV-irradiated water giving a final volume of 45 µL. 5 µL of extracted DNA and 5 µL of extracted DNA diluted in 1 in 10 were added to two separated aliquots of this mixture and the reaction was heated to 94°C for 3 min, followed by 25–35 cycles of 94°C for 30 s, 63°C for 1 min, and 72°C for 1 min. A final extension was done at 72°C for 5 min; PCR reactions were electrophoresed through a 2% agarose gel containing 2 µL of ethidium bromide at 500 nM and bands were visualized by UV transillumination. Positive PCR reactions were sequenced with ABi PRISM Big-dye sequencing kit, and analyzed on the ABI 377 genetic analyzer (PE Applied Byosystem). Datasets were analyzed to obtain phylogenetic analyses and bacterial identification.

Samples were stained with Gram according to already described technique, in previous chapters.

3. Results

20 samples from 10 successive thoracotomies were obtained from 10 lung-cancer tissues and 9 samples from 10 neighboring healthy lung tissues. All patients, of whom 6 were men, had a median age of 62.2 (1SD 9.1) years. The tumors were 8 adenocarcinomas and 2 squamous cell carcinomas. Characteristics of these cases such as TNM and type of surgery can be seen in Tables 10/1 and 10/2. Two patients (cases 72 and 74) were prepared for a left lobectomy, but during the surgery procedure, the tumors could not be removed in their entirety and pneumonectomy was ruled out due to their poor respiratory lung function. All tumoral samples showed abundant intracellular Gram-positive cocci and after mechanical compression and crushing of cells, these cocci could be seen outside the cells in pairs and/or chains.

Two cases (numbers 69 and 71) corresponded to pulmonary metastases of gastric and endometrial adenocarcinomas, respectively, and presented crowded cocci inside malignant cells, as it occurred in pulmonary renal metastases (Chapter 7). See Figures 10/2 and 10/7.

All samples (neoplasic and non-tumoral tissue) were sequenced for 16S DNA, but only 6 tumors and 5 normal lung samples had a combination of MALDI- TOF MS and 16S DNA sequencing for a direct identification of sample concentration (Tables 10/3 and 10/4).

Only 2/20 tumoral and 1/9 non-tumoral samples gave a reliable bacterial identification [χ^2 (Yates' correction) = 0.4 no significant differences]. In the remaining samples no usable sequence was detected above the background.

4. Discussion

The 20 tumoral samples showed many intracelluar Gram-positive cocci (Figures 10/1 to 10/4), which, after cellular compression and crushing, could be seen abundantly outside neoplasic cells (Figures 10/5 and 10/6). These cocci were usually in pairs (diplococci) (Figures 10/7 to 10/13) or in short chains (Figures 10/14 to 10/25 and Figures 8/71 to 8/87) or, less frequently, as long chains (Figures 10/26 to 10/29). Thus, they could be recognized as *Streptococcus sp*. Despite the large number of cocci, two powerful methods (MALDI-TOF mass spectrometry and 16S rRNA gene (or 16S DNA) sequencing failed to show any signal above background in 17 samples.

Basically, a mass spectrometer consist of three phases. In the first phase, the molecules (proteins) to be analyzed are ionized (ion source). In the second phase, the ionized molecules are then accelerated and the different individual ions are separated on the basis of size and charge. The third phase is a detector, which records the signal from all ions. The spectra are analyzed by standard pattern-matching process for a reliable identification (NCB Inn database).[5][6][7]

The inability of MALDI-TOF mass spectrometry to identify all samples may be explained by various reasons. Technical problems such as sensitivity and calibration play an important role, especially during pre-analysis preparation. Protein extraction with alcohol gave better results than the direct analysis of the sample in the matrix.[4][8]

5 Jurinke C, Oeth P, Van den Boom P. *Maldi- Tof mass spectrometry. A versatil tool for high- performance DNA analysis.* Mol Biotech 2004; 26: 147-63.

6 Lay J D. *Maldi- Tof mass spectrometry of bacteria.* Mass Spect Rev 2001; 20: 172-94.

7 Cherkaoui A, Emonet S, Fernandez J, Schorderet D, Schrenzel J. *Evaluation of matrix-assisted laser desorption ionization - Time of flight mass spectrometry for identification of Beta- hemolitic streptococci.* J Clin Microbiol 2011; 49: 3004-5.

8 Williams TL, Andrzejwski D, Lay JO, Musser SM. *Experimental factors affecting the quality and reproductibility of Maldi- Tof mass spectra obtained from whole bacteria cells.* J Am Soc Mass Spect 2003; 14: 342-51.

Another important cause of identification failure is contamination during sample handling, due to the reduction of intensity of important bacterial signals by the signals from human products, such as alfa-defensins and keratins, which can exist in important quantities in tumoral cells of the lower respiratory tract [9]. Moreover, some bacteria that produce tiny or mucoid colonies may yield a small amount of proteins, insufficient for an ideal spectrum; an example of this phenomenon streptococci of the Viridans Group.[4]

However, the most likely cause of lack of signal is the unbalanced quantity and complexity of a tumoral mass, compared to that of bacterial cells. In fact, despite the attempts to concentrate bacterial load, the samples had a great predominance of tumoral cells and their products. Most of the MALDI-TOF MS bacterial tests are based on positive bacterial cultures and, only in some occasions, directly on certain organic media, such as urine. But these media do not have the heterogeneous structure of tumor tissue.

Thus, 29 samples (including 11 without MALDI-TOF MS identification) were processed with 16S DNA sequencing for bacterial identification. Bacterial DNA consists of unique circular DNA. Ribosomes are cell components that synthetize proteins, and they are composed of several proteins and RNA. Bacteria, as prokaryotes, have smaller ribosomes (70S) than do eukaryotes (80S), which ribosomes contain two subunits: a large one (50S) and a small one (30S). These numbers indicate sedimentation rates in Svedberg units. The small subunit (30S) contains 16S rRNA gene and about 20 proteins. The 16S rRNA gene is coded by the gene rrs. The 16S rRNA gene sequence has one series or more of aminoacids that are specific to a phylogenetic species and are used to identify bacterial species. When they present more than 70% similarity, bacteria belong to the same species; similarity of less than 50% indicates different species. Values between 50% and 70% indicate closely related species. 16S rRNA PCR gene is a powerful tool in the routine diagnostics in clinical microbiology laboratories, where samples are bacteria isolated from cultures [10] [11] [12] [13]. In such circumstances, 16S sequencing successfully identified 98% of environmental isolates.[14] [15]

Nevertheless, bacteria identification is not always possible, as occurred in 26 of our samples. Several factors can explain this lack of identification, such as the database (Gene Bank), which is not peer-reviewed and its quality is not optimum; the lack of universally accepted criteria for 16S gene-sequence based identification for the level of sequence homology required in the studied sample, and the fact that designation of genus and species is under debate.[16] But the most important causes for low or not signal DNA sequence are:

1. Fungal samples can be processed as bacterial samples. In all our cases, we did a wet mount microscopic examination to rule out this possibility. Data from this laboratory (not shown here) on 133 post-surgery specimens (75 from tumors and 58 from neighboring normal lung tissue), cultured onto Sabourand agar and Sabourand with antibiotic media, at room temperature and at 37°C, showed that after 4 weeks, only two fungi grew: *Aspergillus nidulans* and *Scopulariopsis brumptii*. Thus, fungi were not responsible for the failure in identification.

2. Other situations that do not produce analyzable spectra are changes within the 16S ribosomal primer regions that affect the ability of PCR primers to anneal to the template.

[9] Carter D, Eggleston J. *Tumors of the lower respiratory tract. Second Series.* Washington DC Armed Forces Institute of Pathology. 1979: 20-1

[10] Chen CC, Teng LJ, Chang TC. *Identification of clinically relevant viridans group streptococci by sequence analysis of the 16S - 22S ribosomal DNA spacer region.* J Clin Microbiol 2004; 42: 2651-7.

[11] Clarridge JE. *Impact of 16S rRNA gene sequence analiysis for identification of bacteria on clinical microbiology and infectious diseases.* Clin Microbiol Rev 2004;14: 840-62.

[12] Drancourt M, Bollet C, Carlioz R, Martelin R, Gayral JP, Raoult D. *16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentificable bacteria isolates.* J Clin Microbiol 2000; 38: 3623-30.

[13] Patel JB. *16 S r RNA gene sequencing for bacterial pathogen identification in the clinical laboratory.* Mol Diagn 2001; 6: 313-21.

[14] Mignard S, Flandrois JP. *16S r RNA sequencing in routine bacterial identification: a 30- month experiment.* J Microbiol 2006; 67: 574-81.

[15] Woo PCY, Ng KHI, Lan SKP, Yip K-T, Fung AMY, Leung KW, Tam DMW, Que T-L, Yuen KY. *Usefulness of the Micro seq 500 16 S ribosomal DNA- based identification system for identification of clinical significant bacteria isolates with ambiguos biochemical profiles.* J Clin Microbiol 2003; 41: 1996-2001.

[16] Boudewijns M, Bakkers JM, Sturm PDJ, Melchers WJG. *16 S r RNA gene sequencing and the routine clinical microbiology laboratory: a perfect mariage?* J Clin Microbiol 2006; 44: 3469-70.

3. The most likely causes for low or no signal DNA are PCR inhibitors in the sample that interact with DNA polymerases or cofactors such as magnesium. The list of PCR inhibitors is long: poyssaccharides, urea, humic acids, etc. Hemoglobin and collagen, important inhibitors, can be present in important quantities in lung tumors.[17] In neoplasms, cocci are mixed with a high quantity of DNA of tumoral cells. In consequence, the ratio between target DNA sequence to be amplified and the non-target DNA or *"burden DNA"* is very low, and this is a known inhibitor factor of PCR.

4. Other factors can also play a role in the lack of spectra, such as type or concentration of specific primers.[18][19]

In summary, low quantity of bacterial proteins and/or high levels of contaminants, together with the complexity of the samples, result in a weak spectrum and identification failure. New efforts are necessary to improve the isolation of these streptococci or the use of other diagnostic techniques.

[17] Moreira D. *Efficient removal of PCR inhibitors using Agarose- embedded DNA preparations.* Nucl Acids Research 1998; 26: 3309-10.

[18] Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom S J, Wade WC. *Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16 S r RNA.* Appl Environ Microbiol 1998; 64: 795-9.

[19] Lane DJ. *16S / 23S rRNA sequencing. In Nuclei acid techniques in bacterial systematics.* ed. Stackebrandt E, Goodfellow M. Chicester John Wiley Sons: 115-75.

Table 10/1. CHARACTERISTICS OF 10 CASES: GENOTYPING PROCEDURES.

Nº	AGE (years)	GENDER	TYPE OF TUMOR	STAGING			PRIMARY TUMOR		TEST	TYPE OF LUNG RESECTION
				T	N	M	Φ MAXIMAL (cm)	AREA (cm^2)		
62	54	MALE	SQUAMOUS CELL	1	0	X	2.5	5	SYTO PLUS PROPIDIUM IODIDE GENOTYPIC TEST	NEUMONECTOMY
64	74	MALE	ADENOCARCINOMA	2	1	X	4	14.8	SYTO PLUS PROPIDIUM IODIDE GENOTYPIC TEST	LOBECTOMY
69	64	MALE	METASTATIC GASTRIC ADENOCARCINOMA	3	0	X	8.0	56	GENOTYPIC TEST	LOBECTOMY
70	76	MALE	ADENOCARCINOMA	2	0	1	5.5	22	GENOTYPIC TEST	LOBECTOMY
71	65	FEMALE	METASTATIC ENDOMETRIAL ADENOCARCINOMA	1	0	1	2.8	5.6	GENOTYPIC TESTS	LOBECTOMY
72	56	MALE	SQUAMOUS CELL	1	0	0	2.5	5.5	GENOTYPIC TESTS	NO RESECTION
73	52	FEMALE	ADENOCARCINOMA	2	0	X	3.5	7	GENOTYPIC TESTS	LOBECTOMY
74	52	FEMALE	ADENOCARCINOMA	4	0	0	2.0	4	GENOTYPIC TESTS	NO RESECTION
75	58	MALE	ADENOCARCINOMA	3	0	0	8.0	56	GENOTYPIC TESTS	NEUMONECTOMY
76	71	FEMALE	ADENOCARCINOMA	1	0	0	3.0	9	GENOTYPIC TESTS	LOBECTOMY

Table 10/2. SOCIODEMOGRAPHICS AND TUMOR CHARACTERISTICS.

AGE (years)	MEAN ± 1SD. (MAXIMUM, MINIMUM)	62.2 ± 9.1 (52-76)		
GENDER	FEMALE	4 (40.0%)		
	MALE	6 (60.0%)		
TYPE OF TUMOR	ADENOCARCINOMA	6 (60.0%)		
	SQUAMOUS- CELL CANCER	2 (20.0%)		
	METASTATIC CANCER	2 (20.0%)		
TNM DESCRIPTOR		T	N	M
	0		9 (90.0%)	4 (40.0%)
	1	4 (40.0%)	1 (10.0%)	2 (20.0%)
	2	3 (30.0%)		
	3	2 (20.0%)		
	4	1 (10%)		
	X			4 (40.0%)
TYPE OF LUNG RESECTION	NO RESECTION	2 (20.0%)		
	LOBECTOMY	6 (60.0%)		
	NEUMONECTOMY	2 (20.0%)		

Table 10/3. GRAM STAINING: COCCI DETECTED IN GENOTYPIC SAMPLES.

GRAM STAINING	TUMOR	NON TUMORAL LUNG	TOTAL
POSITIVE	20	2	**22**
NEGATIVE	0	7	**7**
TOTAL	**20**	**9**	**29**

Sensitivity: *100.0 % 95% CI (85%-100%)*
Specificity: *77.7 % 95% CI (64%-94%)*
OR: *Undefined*
χ^2 ***(correction Yates):*** *16.6 (P = 0.000)*

Table 10/4. SAMPLES AND RESULTS FROM MALDI-TOF MASS SPECTROMETRY (MS)AND 16S rRNA GENE SEQUENCING.

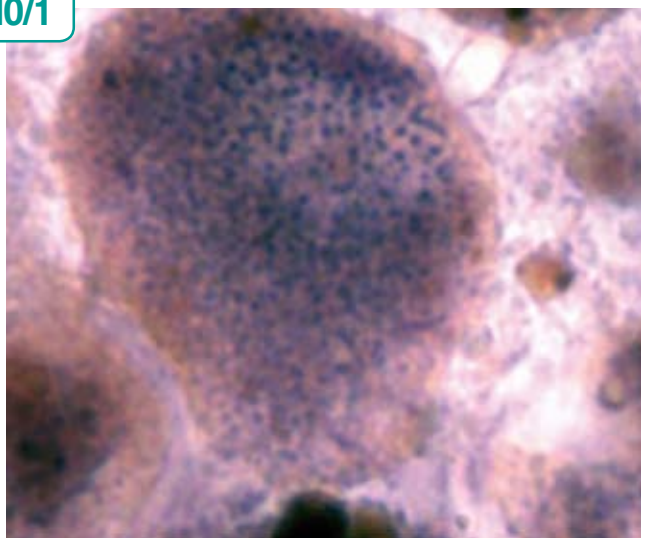
CASE NUMBER	TUMOR			NON TUMORAL LUNG		
	SAMPLES NUMBERS	METHODS	IDENTIFICATION	SAMPLES NUMBERS	METHODS	IDENTIFICATION
62	1	16S rRNA GENE SEQUENCING	STAPHILOCOCCUS EPIDERMIS	2	16S rRNA GENE SEQUENCING	NO IDENTIFICATION
64	3	16S rRNA GENE SEQUENCING	NO IDENTIFICATION	4	16S rRNA GENE SEQUENCING	ACINETOBACTER SP
69	5 to 12	16S rRNA GENE SEQUENCING	NO IDENTIFICATION	13	16S rRNA GENE SEQUENCING	NO IDENTIFICATION
70	14 to 17	16S rRNA GENE SEQUENCING	NO IDENTIFICATION	18	16S rRNA GENE SEQUENCING	NO IDENTIFICATION
71	19	16 SrRNA GENE SEQUENCING MALDI-TOF MS	MORAXELLA OSLOENSIS	20	16S rRNA GENE SEQUENCING MALDI-TOF MS	NO IDENTIFICATION
72	21	16S rRNA GENE SEQUENCING MALDI-TOF MS	NO IDENTIFICATION	22	16S rRNA GENE SEQUENCING MALDI-TOF MS	NO IDENTIFICATION
73	23	16S rRNA GENE SEQUENCING MALDI-TOF MS	NO IDENTIFICATION	24	16S rRNA GENE SEQUENCING MALDI-TOF MS	NO IDENTIFICATION
74	25	16S rRNA GENE SEQUENCING MALDI-TOF MS	NO IDENTIFICATION	26	16S rRNA GENE SEQUENCING MALDI-TOF MS	NO IDENTIFICATION
75	27	16S rRNA GENE SEQUENCING MALDI-TOF MS	NO IDENTIFICATION	0	NO SAMPLE	-
76	28	16S rRNA GENE SEQUENCING MALDI-TOF MS	NO IDENTIFICATION	29	16S rRNA GENE SEQUENCING MALDI-TOF MS	NO IDENTIFICATION

Gram staining (×2000).

FIGURES 10/1 TO 10/29

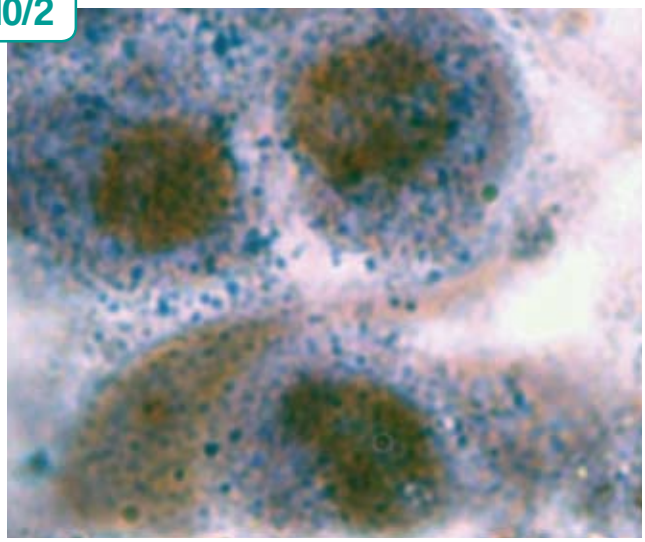
→ **Intracellular Gram-positive cocci, most of them inside intranuclear tumoral cells** (Figures **10/1** to **10/4**)

10/1



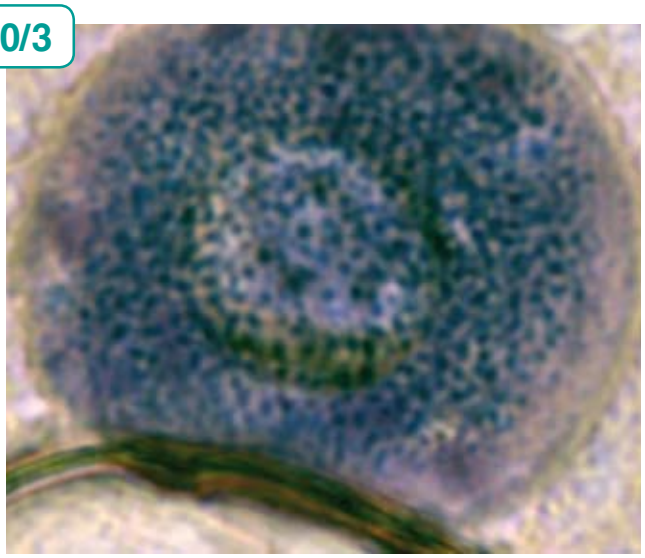
Squamous cell carcinoma
(case 72. Sample number 21)

10/2



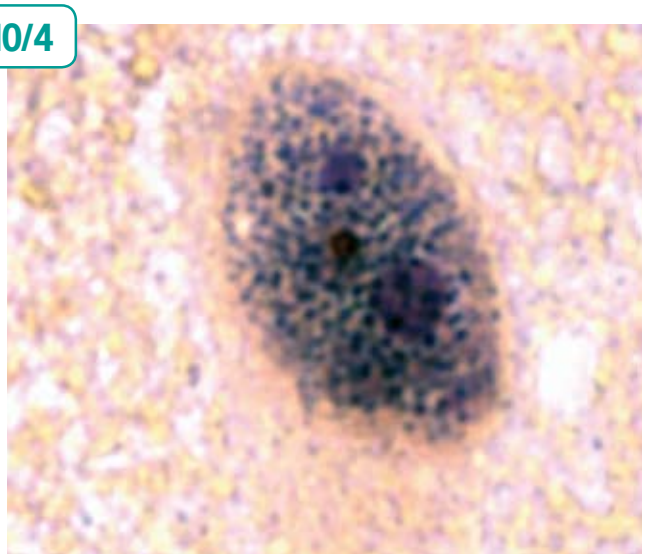
Pulmonary metastases from endometrial adenocarcinoma
(case 71. Sample number 19)

10/3



Lung adenocarcinoma
(case 74. Sample number 25)

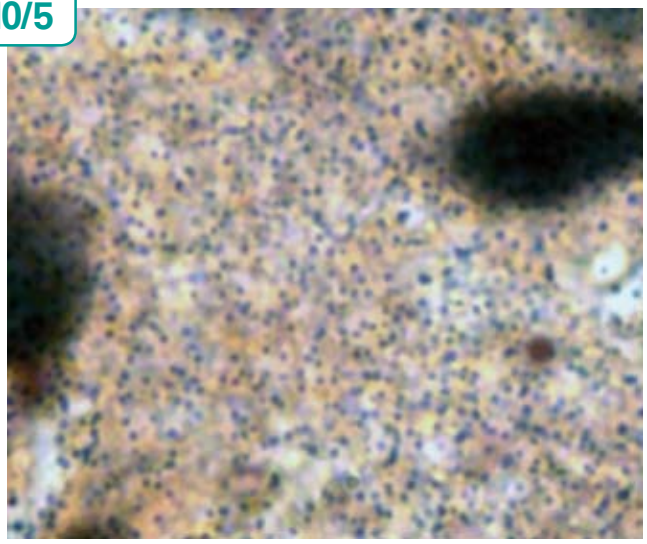
10/4



Lung adenocarcinoma
(case 76. Sample number 28)

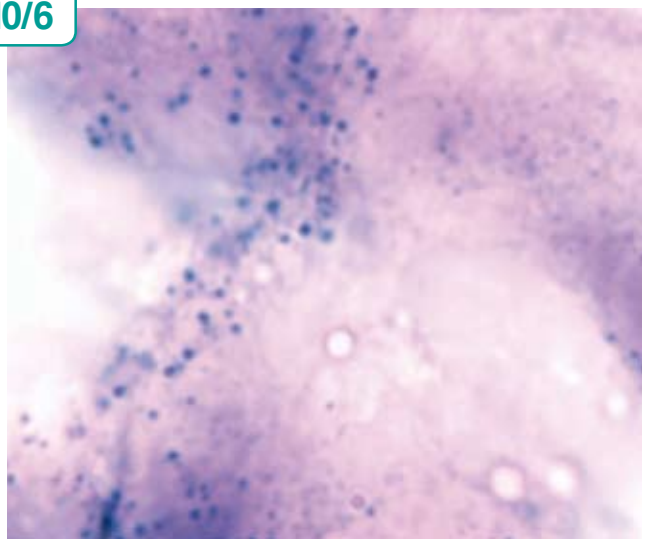
→ **Extracellular Gram-positive cocci after mechanical compression and crushing neoplasic cells** (Figures **10/5** to **10/6**)

10/5



Lung adenocarcinoma
(case 76. Sample number 28)

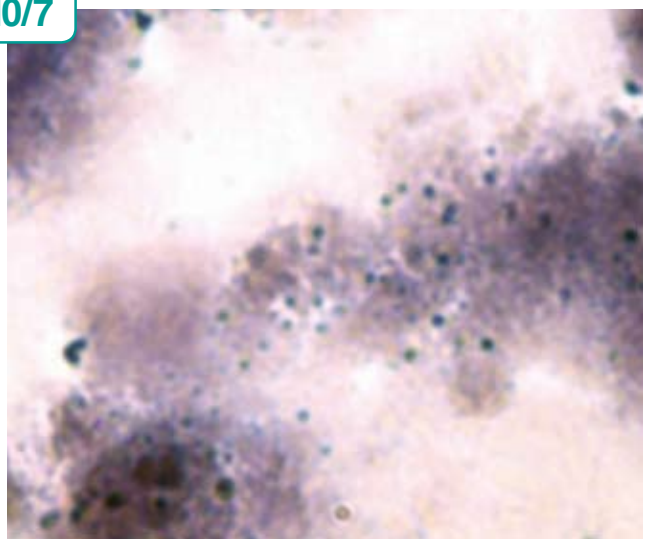
10/6



Lung adenocarcinoma
(case 73. Sample number 23)

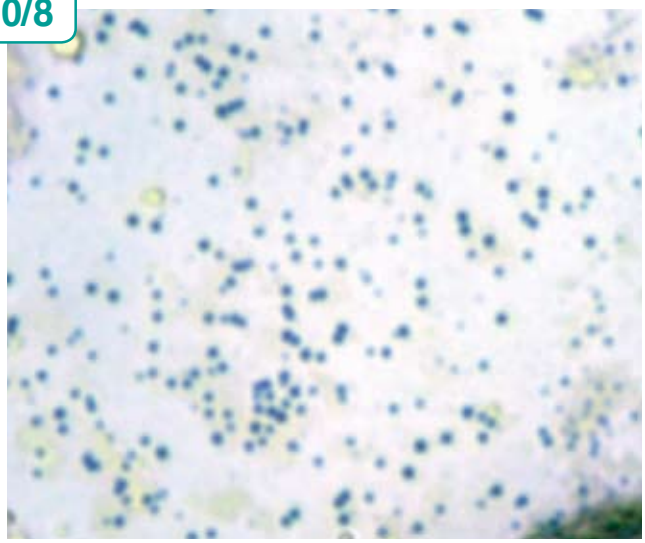
→ **Sparse cocci are usually present in pairs or tetrads** (Figures **10/7** to **10/13**)

10/7



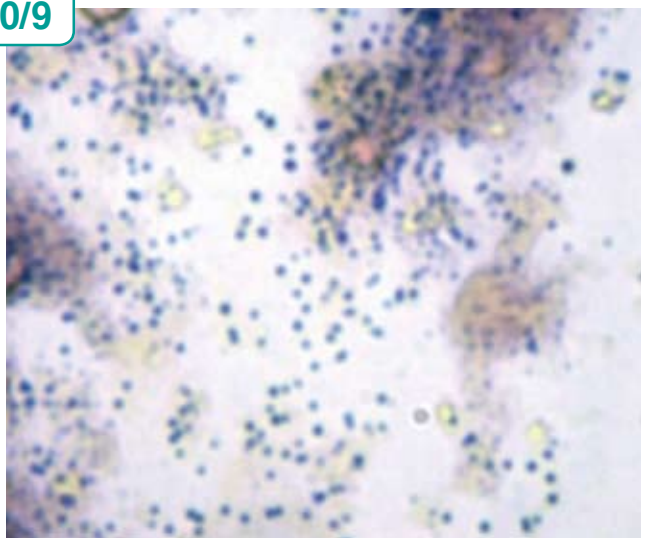
Pulmonary metastases from gastric adenocarcinoma
(case 69. Samples 5 to 12)

10/8

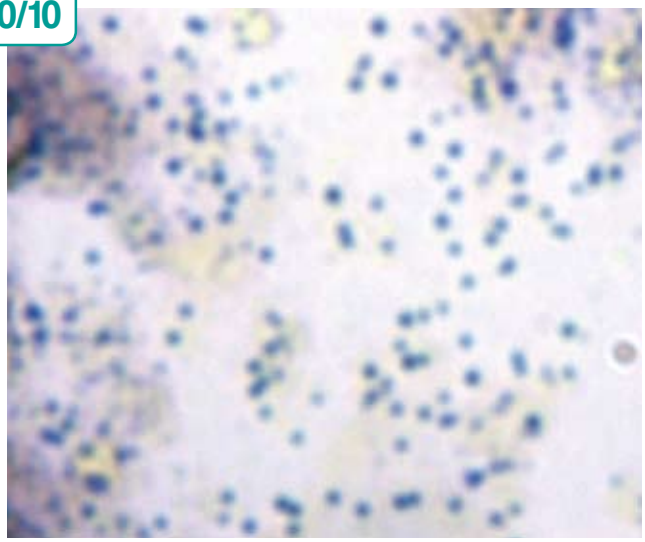


Lung adenocarcinoma
(case 75. Sample number 27)

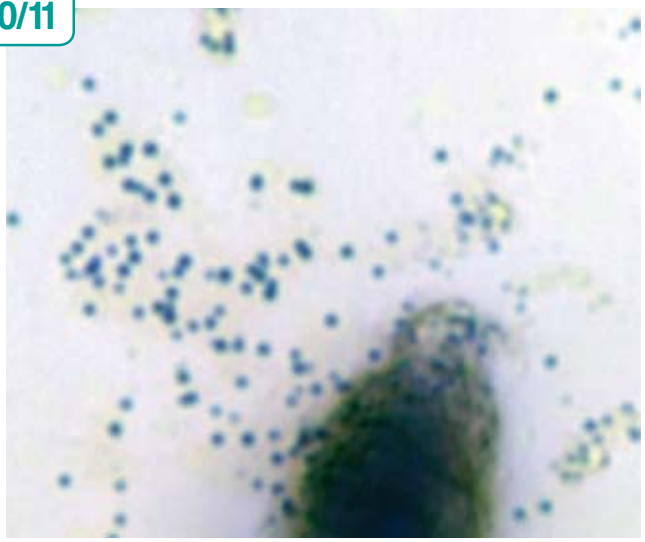
10/9



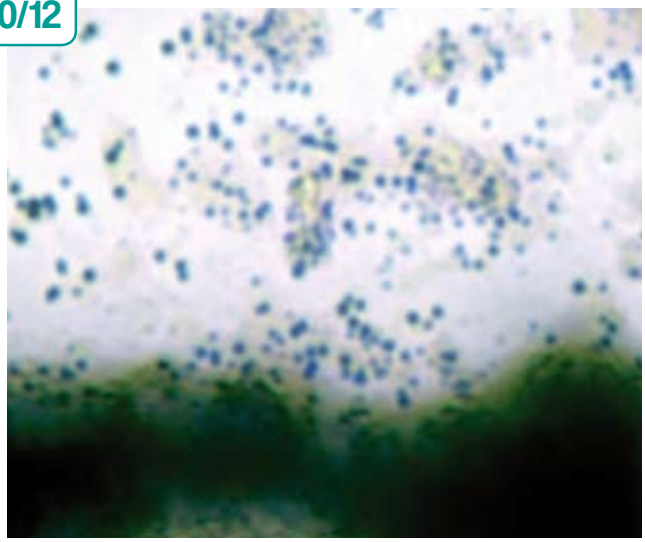
10/10



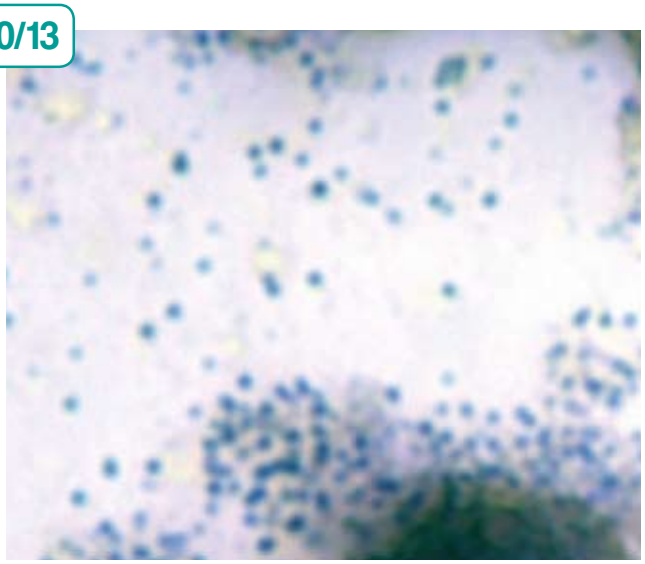
10/11



10/12



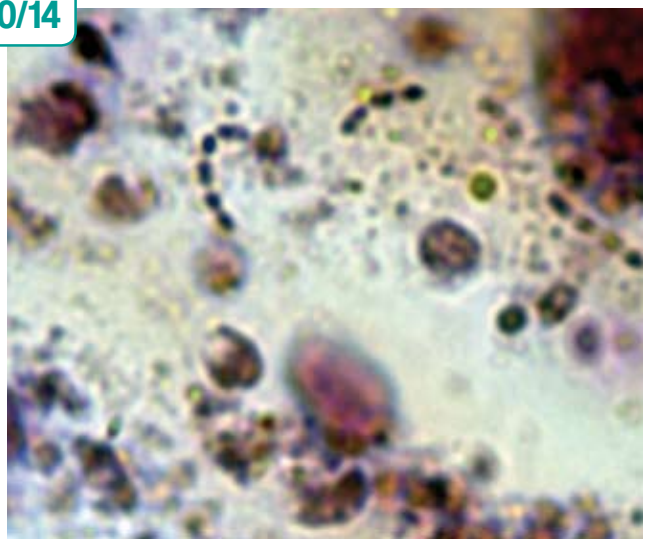
10/13



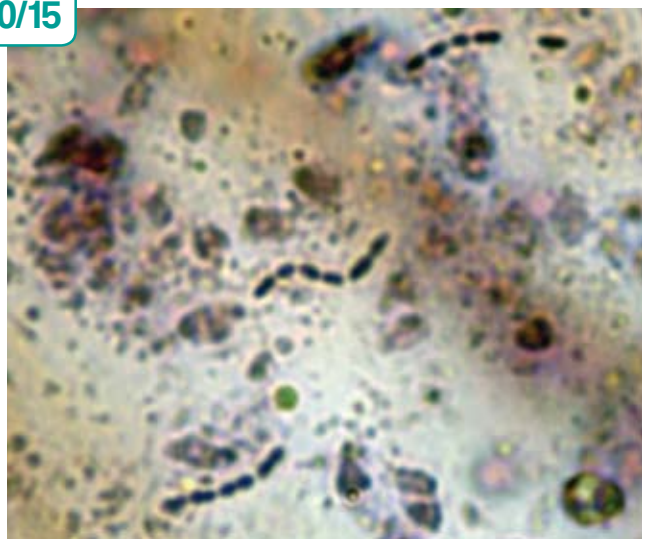
Extracellular cocci distribution. General view
(case 76. Sample number 28).

→ **Cocci presentation as short chains (cases 62, 64 and 70. Samples numbers: 1, 3 and 14 to 17)** (Figures **10/14** to **10/25**)

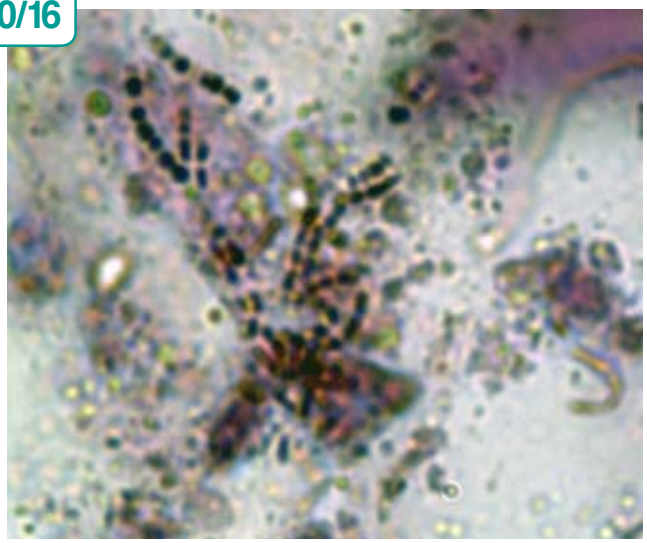
10/14



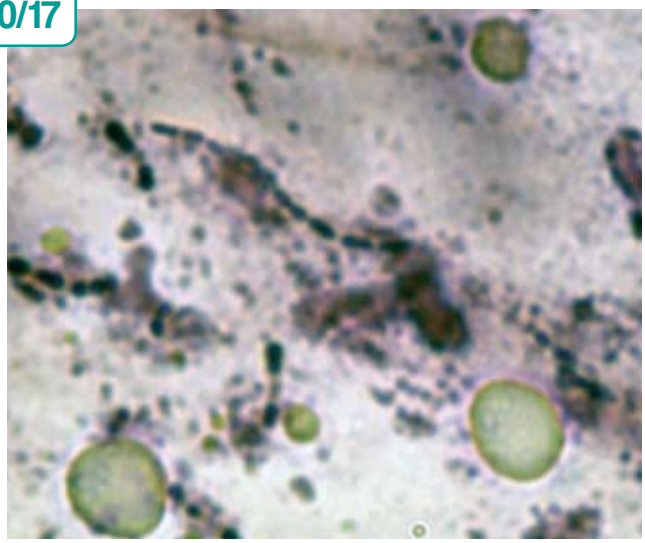
10/15



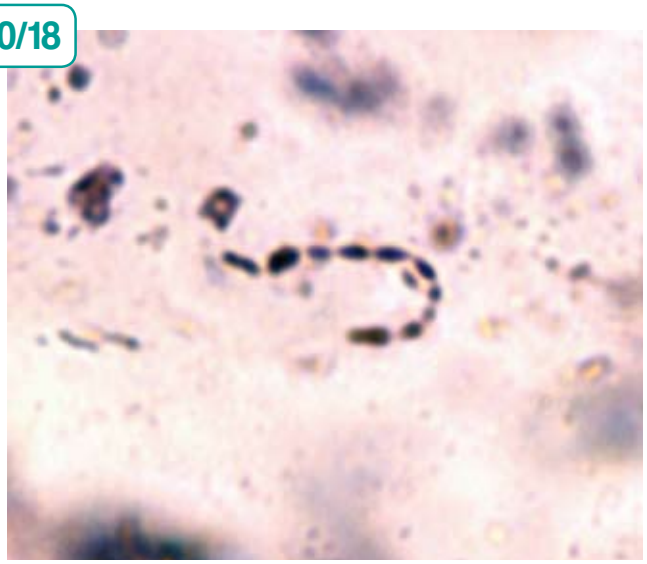
10/16



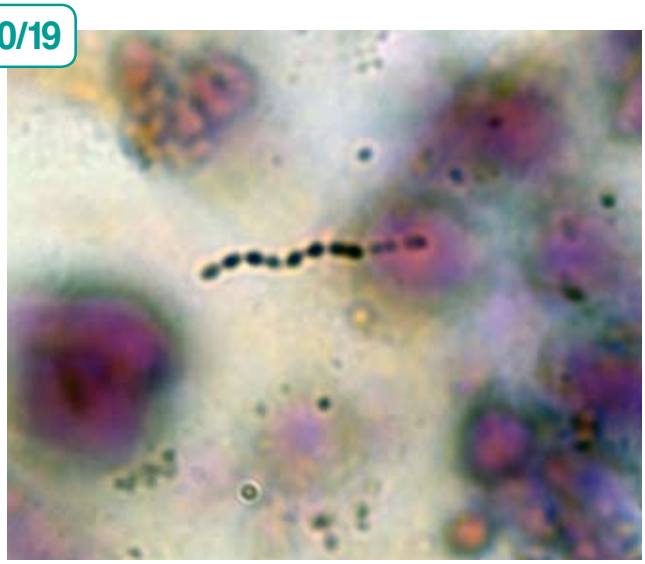
10/17



10/18

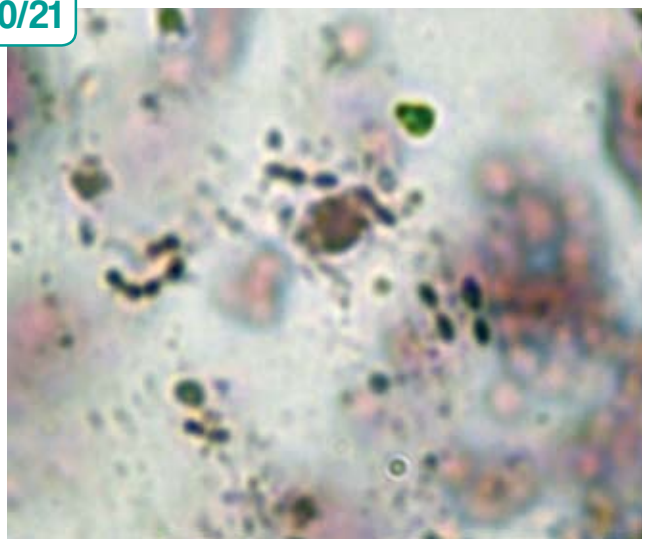


10/19



10/20

10/21



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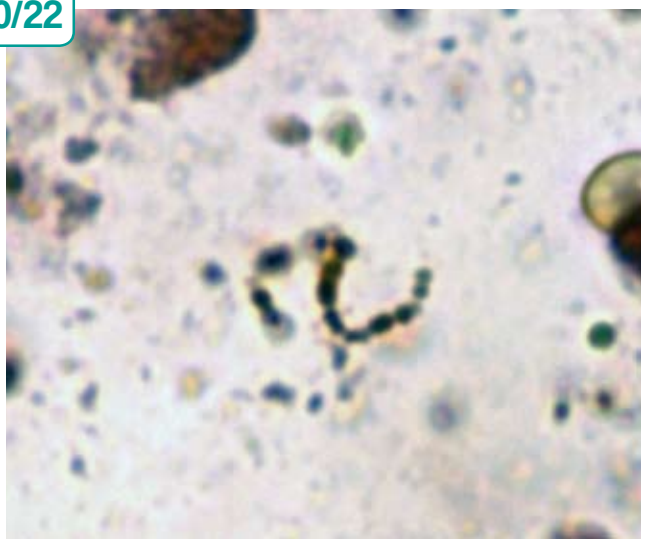
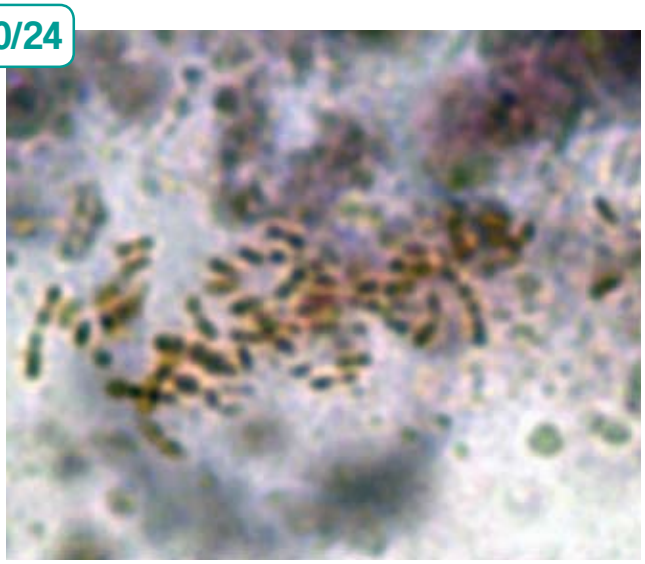
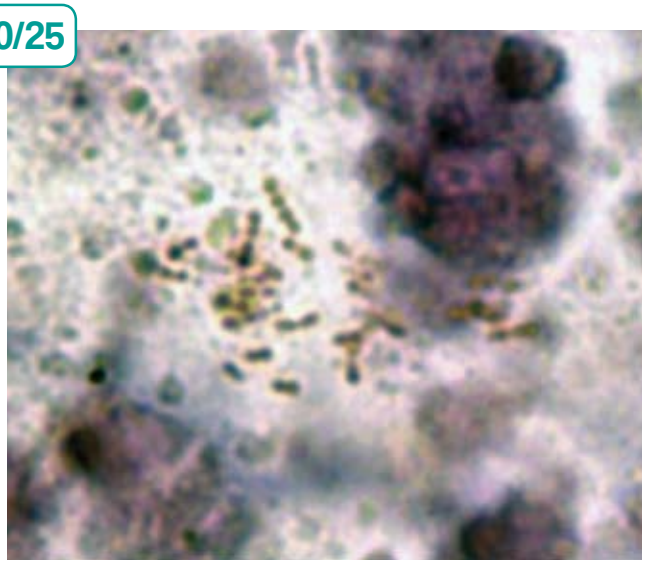


10/23

10/24

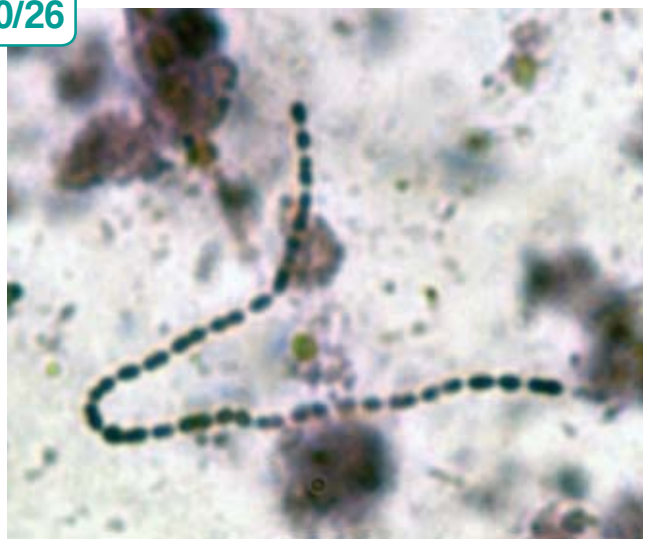


10/25

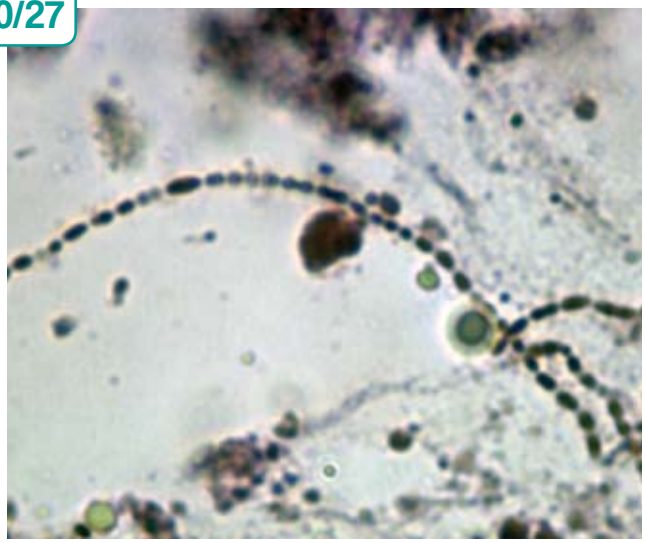


→ **Cocci presentation as long chains (cases 62 and 70. Sample numbers: 1 and 14 to 17)** (Figures **10/26** to **10/29**)

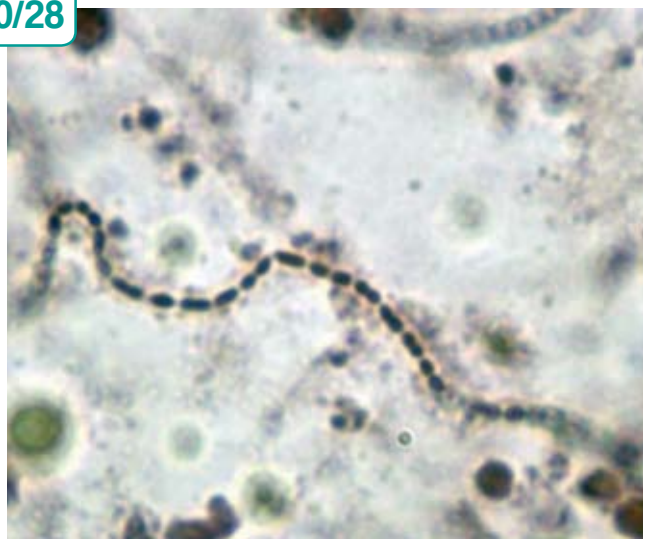
10/26



10/27



10/28



10/29

CHAPTER

11 ENDOSYMBIONTS: A COMMON SETTLER

1. Introduction

After the first discoveries of microorganisms by Leeuwenhoek (1632-1723), Spallanzani (1729-1799), and Pasteur (1822-1885), their invasive and pathogenic role in humans was recognised. In 1856, Müller observed living bacteria in the nuclei of ciliates, and since then, this type of association has been widely reported, especially in protozoa and insects.[1]

2. Definition

Endosymbiosis means a close association between different organisms that can have several forms, such as parasitism, commensalism, and mutualism, depending on the level of benefit between the host and the invader. The type of relationship can change over time, and it is accepted that, in bacteria, invasion is not accidental but is in fact a permanent colonization inside the host cell, transmitted during cellular division to daughter cells. In the case of highly infectious bacteria, these must find a way to leave the cells and invade new hosts. These organisms can be classified according to the part of the cell that they colonize: endonuclear symbionts are present in the nucleus, and endocytoplasmic symbionts in the cytoplasm.[2]

3. Examples of bacterial endosymbionts

Protozoa are the most extensively studied hosts. During the last century, at first, germs were named *"particle"* followed by a Greek letter such as alpha, kappa, etc.[3][4] When their bacterial nature was proven, several new names were given, sometimes to the same species, despite many efforts to create a standard nomenclature.[4][5] For instance, the number of names for bacteria found within amoebas is about 30.[6] This has created many difficulties in the classification of these organisms. 16S rRNA gene sequencing analysis greatly improves the phylogenetic classification of endosymbionts,[7] but these improvements can cause new problems. An example is the genus *Caedibacter*, a common protozoa symbiont. It is polyphyletic and there are at least two phylogenetically different bacterial species with identical morphology within the genus; one relates to Rickettsiales (alphaproteobacteria) and the other to *Francisella tularensis* (gammaproteobacteria).[8][9]

1 De Kruif P. *Cazadores de microbios.* Barcelona: Salvat Editores SA, 1986: 143.
2 Gortz Hd. *Endonuclear symbionts in ciliates.* In Rev Cytol 1983; 14 (suppl): 145–75.
3 Preer Jr. *Microscopically visible bodies in the cytoplasm of the "killer" strains of Paramecium aurelia.* Genetics 1950; 35: 344–62.
4 Preer Jr, Stark P. *Cytological observations on the cytoplasmic factor " Kappa" in Paramecium aurelia.* Exp Cell Res 1953; 5: 478–91.
5 Schmidt Hj, Gortz Hd, Quackenbush Rl. *Caedibacter caryophila sp. Nov., a killer symbionts inhabiting the macronucleus of Paramecium caudatum.* Int J Syst Bacteriol 1987; 37: 459–62.
6 Preer Jr, Preer Lb. *Revival of names of protozoan endosymbionts and proposal of Holospora caryophila nom.* nov. Int Syst Bacteriol 1982; 32: 140–1.
7 Manz W, Amann R, Ludwig W, Wagner M, Schleifer Kh. *Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions.* System Appl Microbiol 1992; 15: 593–600.
8 Pond Fr, Gibson I, Lalucat J, Quackenbush Rl. *R-body-producing bacteria.* Microbiol Rev 1989; 53: 25–67.
9 Beier Cl, Horn M, Michel R, Schweikert M, Gortz Hd, Wagner M. *The genus comprises endosymbionts of Paramecium spp. realted to the Rickettsiales (Alphaproteobacteria) and to Francisella tularensis (Gammaproteobacteria).* Appl Environ Microbiol 2002; 68: 6043–50.

4. Cell penetration

The mechanism of entrance of bacterial endosymbionts into the host cell is speculative. Organisms could penetrate eukaryote cells by active penetration, causing cell membrane defects that are not lethal for the cell, or, what seems more likely, by phagocytosis. In both cases, infectious organisms overcome cellular defenses, mainly lysosome activity, and spread into the cell cytoplasm. Entrance into the nucleus happens sometimes very quickly, ie *Leptomonas karyophilus* reaches the nucleus of *Paramecium trichium* in 20–30 min.

5. Types of endosymbiosis

Most bacteria live in poor conditions inside eukaryotic cells because of their restrictive environment: hypoxia, lytic enzymes, etc. Therefore, accidental invasion usually ends quickly with death of the host cell or of the endosymbiont. Permanent colonisation requires a high degree of adaptation, and, in some cases, a type of benefit for one or both of the organisms. The balance host-guest is not always stable, and can change with physiological metabolic rates.

6. Effects on host nuclei

Endonuclear symbionts are extensively studied in protozoa, their action in the host nuclei is different if the nucleus is in interphase or in the mitotic cycle.

6A. Effects on nuclei in interphase

Three types of effects can be seen depending on the adaptive degree and infectivity of the bacteria:

1. The shape and size of the infected nucleus is not modified. This situation occurs when symbionts are weak or not infectious, small, and scarce, and when they do not fill the nucleus completely, ie MS 2 in P caudatum or the small bacteria in *Neobursidum gigas* and *Spirostomun ambiguum*.

2. The chromatin is destructed, as occurs with species of *Leptomonas* in *P trichium*.

3. The shape of the infected nucleus is modified and its size increases as a result of symbiont overpopulation and of the destruction of microtubules and microfilaments of the nuclear skeleton, leading to nucleus deformations. In Vorticella, the macronucleus swells after infection, its extreme hypertrophy makes it loose its elongated form, and it becomes a large ellipsoidal body. Another example is the extreme hypertrophy that occurs in the macronucleus of P caudatum, which bears many Kappa endonuclear symbionts.

   The extent of nuclear hypertrophy depends on the metabolic conditions of the moment. In conditions of starvation, the macronuclei of P caudatum containing Holospora obtusa are hypertrophied. However, in fast growing cells the macronuclei are of normal size because the speed of division of the nuclei is faster than that of the symbionts, which are thus unable to enter the new macronucleus. This can lead, in some cases, to the clearance of the parasite.[10]

6B. Effects on nuclei in mitosis

Bacteria can induce or inhibit mitosis, depending on the severity of infection.[11] Highly infectious organisms, such as Genus *Holospora*, take advantage of the host's mitotic cycle to spread to daughter cells (reproductive stage), and to be released and thus propagate to other cells (infectious stage).

[10] Schmidt HJ, Freiburg M, Gortz HD. *Comparison of the infectious forms of the bacterial endonucleosymbionts, Holospora elegans and H. obtusa, from the ciliate Paramecium caudatum*. Microbios 1987; 49: 189–97.

[11] Preer Lb. *Alpha, an infectious macronuclear symbiont of Paramecium aurelia*. J Protozool 1969; 16: 570–8.

6C. Effects of endosymbionts on viability of host cells

Some symbionts have no effect on the host cell's viability, such as the Gram-negative bacteria inside P bursae. Others shorten the host's lifespan, as is the case of Wolbachia, a parasite of the mosquito *Aedes albopictus*; and in some cases they can even cause death, like the case of bright Kappa particles in P caudatum.[12][13]

Endosymbionts have had a strong influence on the evolution of arthropods, influencing their reproduction and supplying them with nutrients. An example is the aforementioned Wolbachia, which induces the Insulin/insulin-like growth factor (IGF) signalling in Drosophila, causing multiple effects on its growth, development, metabolic homeostasis, fecundity, and lifespan of the insect.[14][15] The mutualistic relation between bacteria and insects is not limited to the supply of essential nutrients to the host. For example, reed beetles (*Chrysomelidae, Donacliinae*) use a secretion produced by their symbionts *Candidatus macropleicola*, an Enterobacteriaceas, to create a water-tight cocoon for their larvae.[16] The endosymbiont Wolbachia is maternally inherited, and when colonizing the pseudoscorpion *Cordylochernes scorpioides*, it favours the killing of male individuals. Male killing is an extreme form of manipulation of sex ratio: by self-sacrificing Wolbachia in males, the endosymbiont increases its chances of transmission through females.[17]

Soldo and colleagues have shown that, when paramecia of the genus *Paramecium aurelia* containing endosymbionts are placed in a poor medium, they die. If the paramecia were examined just before their death, the investigators found that endosymbionts had increased to exceeding high numbers. The strong suspicion that endosymbionts were the cause of dead, rather than the result of poor medium, was reinforced by the fact that endosymbiont-free strains multiplied perfectly well in the same medium.[18]

6D. Toxic effects on other cells

A lot of endosymbionts produce and liberate toxins into the medium in which they live. These toxins are generally harmless to their host, but they can cause injuries or death in certain sensitive cells. These actions can occur without the endosymbionts leaving their hosts. This killer trait was discovered in 1938 by Sonneborn in endosymbionts, and the knowledge of its nature and physiological mechanisms has improved since. A classic example is the effect of Kappa symbionts inside *P aurelia*. The killer trait is associated with the presence of bright inclusions that can be seen with light microscope, using bright phase contrast. These bright inclusions are inside the kappa particles, and are called refractory (R) bodies. The kappa particles containing these inclusions are called bright Kappas. In 1953, Preer and colleagues [18] showed that bright Kappa particles are toxic, and that their toxic activity was entirely associated with bright R bodies released into the medium.

[12] Suh E, Mercer Dr, Fu Y, Dobson Sl. *Pathogenicity of life-shortening Wolbachia in Aedes albopictus after transfer from Drosophila melanogaster.* Appl Environm Microbiol 2009; 75: 7783–88.

[13] Proca-Ciobanu M, Lupascu Gh, Petrovici Al, Ionescu Md. *Electron microscopic study of a pathogenic Acanthamoeba castellanii strains: the presence of bacterial endosymbionts*. Int J Parasitol 1975; 5: 49–56.

[14] Gibson Cm, Hunter Ms. *Extraordinary widespread and fantastically complex. Comparative biology of endosymbiotic bacteria and fungal mutualists of insects.* Ecology Letters 2010; 13: 223–34.

[15] Ikeya T, Broughton S, Alic N, Grandison R, Partridge L. *The endosymbionts Wolbachia increases Insulin/ IGF-like signaling in Drosophila*. Proc Royal Soc Biol Sci 2009; 276: 3799–807.

[16] Kolsch G, Pedersen Bv. *Can the tight co-speciation between reed beagles (col. Chrysomelidae, Donacliinae) and their bacterial endosymbionts, which provide cocoon material, qualify the deeper phylogeny of the hosts?* Mol Phylogen Evol 2010; 54: 810–21.

[17] Koop Jl, Zeh Dw, Bonilla Mm, Zeh Ja. *Reproductive compensation favours male-killing Wolbachia in a live-bearing host*. Proc Royal Soc Biol Sci 2009; 276: 4021–8.

[18] Preer Jr, Preer Lb, Jurand A. *Kappa and other endosymbionts in Paramecium aurelia*. Bacteriol Rev 1974; 38: 113–63.

These bodies are virus-like or phage-like structures that are visible in an electron microscope. These defective phages are highly integrated inside the nuclei of bacterial endosymbionts and are lethal for paramecia that contain non-bright Kappa particles, while conferring resistance to their hosts. Kappa endosymbionts can also provide a positive benefit to their host in other ways than by bringing the advantage to spread in ecological medium. For example, they can give an extra supply of folic acid necessary for the growth of the host.[18]

Pre-lethal symptoms in paramecia sensitive to endosymbionts toxins are classified into four groups: (a) hump killing, in which sensitive cells develop a large blister; (b) spin killing, in which affected cells swim with an opposite rotation to the normal direction; (c) vacuolisation; and (d) paralysis, in which cells cease swimming.

7. Benefit to endosymbionts

Symbionts generally need the host's metabolism for survival. Consequently, only few symbionts have been cultivated successfully outside their host. Currently, 19 bacterial endosymbionts have been identified in amoebae living in different regions and habitats, mainly amoeba of *Acanthamoeba spp.* Spontaneous loss of symbionts from the original amoeba or successful eradication by antibiotics has never been observed, except in the case of *Neoclamydia hartmannella*, confirming the stability of the association. The numerous and strong associations between bacteria and amoeba are likely to be very ancient. Indeed, they may date to pre-Cambrian times, about 700 million years ago, many years before the first mammal appeared on the earth. As a matter of fact, the genome of the symbiont UWE25 is twice as large as the genome of the pathogenic Chlamydia, but several essential biosynthetic pathways are truncated in the symbiont. Consequently, the symbiont UWE25 relies on essential metabolites from the amoeba such as aminoacids, nucleotides, and cofactors.[19]

The bacteria obtain essential nutrients for their growth and division in the host cytoplasm and nucleus. The induction of mitosis helps the perpetuation of the symbionts into daughter cells. For example, in the *Holospora* species, the presence of *H obtusa* is limited to the macronucleus of *P caudatum*, while the *H elegans* is found exclusively in the micronucleus; however, both cycles show common features. The infectious form of *H elegans* in micronucleus, as well as the one of *H obtusa* inside macronucleus, divide and develop into a reproductive form that multiplies by binary fission. When the nucleus divides, the reproductive forms are then distributed into the daughter nuclei. Some bacteria start to grow and develop into the infectious form again, and remain within the separation spindle. Afterwards, the infectious form is released into the external medium, enabling the symbionts to infect symbiont-free paramecia.[10]

Despite of normal mitosis of symbionts-bearing micronuclei, fission anomalies can occur. Sometimes, individual cells with heavily infected micronuclei divide incompletely and form *"monsters"* with unbalanced distribution of nuclei between daughter cells, with both nuclei remaining within one cell, leaving the other cell anucleate.

For symbionts, nuclei are not only the place for adequate nutrition and genetic control but also a shelter from digestion by lysosomal enzymes. Starving cells can digest parts of their cytoplasm, including organelles and probably cytoplasmic symbionts, but not the nuclei. Only a few lytic enzymes have been shown inside nuclei.[2,20]

8. Destruction, inactivation, or inhibition of endosymbionts

Some investigators have been searching how to inactivate or reduce bacterial symbionts without damaging host cells.[20,22] Some chemical agents produce drastic effects in Kappa particles without affecting the paramecium they colonize. Changes in temperature, doses of X-rays, ultraviolet irradiation, and nitrogen mustards generally reduce the amount of Kappa particles, but do not completely remove them. Some of these agents are more toxic to the paramecia than to the guest. Chemical agents block protein synthesis and electron-transport systems and seem to be essential for inhibiting the killing effect of Kappa particles.[20]

[19] Horn M, Wagner M. *Bacterial endosymbionts of free-living amoebae.* J Eukaryot 2004; 51: 509–14.

[20] Gortz Hd. *Endonucleobiosis in ciliates.* Int Rev Cytol 1986; 102: 169–213.

High concentrations (2000 units/mL) of penicillin G result in the lysis of Lambda particles. Penicillin might interfere with transpeptidation involved in the synthesis of peptidoglycans and possibly cause a weakening of the cell wall. Low concentrations of penicillin G (1–2 units/mL) cause Lambda particles to grow without cell division.[21]

Van Wagtendonk and colleagues showed that a concentration of 3200 units/mL of penicillin G resulted in up to 80% loss of Kappa particles after 2 days of exposure, and to a complete loss of Kappas after 3 or 4 days of treatment. Streptomycin (1000 gammas/mL) and dihydrostreptomycin (25–1000 gammas/mL) seemed to have the same action as penicillin G and there were presumed decreases in the amount of Kappa bacteria.

Chloramphenicol (2 mg/mL) completely removed Kappa particles from the paramecia after 9–14 days of treatment, without affecting the population density of the paramecia. Other experiments showed that doses of 0.02–1.25 mg/mL of chloramphenicol applied for up to 3 days, reduced the concentration of Kappa particles without completely eliminating them or having deleterious effects on the paramecia.

Terramycin (51–200 gammas/mL) or aureomycin (10–25 gammas/mL), both applied for 1 day, reduced or eliminated Kappa particles without toxic effects to the paramecia as it occurs with chloramphenicol. Unclear results were obtained with neomycin and 2,6 Diaminopurine.[22][23]

[21] Butzel Hm, Pagliara A. *The effect of biochemical inhibitors upon the killer sensitive system in Paramecium aurelia*. Exp Cell Res 1962; 27: 382–95.

[22] Soldo At, Musil G, Godoy Ga. *Action of penicillin G on endosymbiont Lambda of Paramecium aurelia*. J Bacteriol 1970; 104: 966–80.

[23] Sonneborn Tm. *Kappa and relates particles in Paramecium*. Adv Virus Res 1959; 6: 229–355.

CHAPTER 12

NEOPLASIC MORPHOLOGY AND FUNCTIONAL CHANGES. THE ENDONUCLEAR SYMBIONT MODEL

1. Introduction

The observation of universe allows us to discover cyclic facts and to make predictions by means of physical-mathematical laws, which constitute the basis of science. The living world is more complex, and can only be described through statistical studies. However, biological facts can also be described by biological models recognized in other areas of nature, because among living beings there are also repeated phenomena.[1]

Here, we will see how the prominent neoplastic characteristics are just an expression of the relationship between endosymbionts and parasitized cells, repeating recognized events that occur in other symbionts that live in distant species, such as protozoa or insects.

2. Etiological agent

Tumoral cells are crowded with Gram-positive cocci, with a propensity to grow as single cocci, diplococcic, tetrads, or as short chains. In such cases, cellular division occurs along a single axis, and they grow in chains or pairs, hence the name Streptococcus -from Greek, streptos- meaning easily bent or twisted, similar to a chain. By contrast, Staphilococci divide along multiple axes and generate grape-like clusters of cells. Most streptococci are oxidase -and catalase- negative and many are facultative anaerobes.

Scientific classification of Streptococcus:

Kingdom: *Bacteria*.
Phylum: *Fimicutes*.
Class: *Coccus*.
Order: *Lactobaciliales*.
Family: *Streptococcaceae*.
Genus: *Streptococcus*.[2]

The streptococci are divided into three groups, on the basis of their haemolytic capacity (alpha, beta, and gamma haemolysis). They can also be classified on the basis of the antigenic characteristics of C carbohydrates found on their cell wall, called Lancefield antigens, which are given letter names ranging from A to S. There are more than 30 species of streptococci, but only a few are significant human pathogens.

The cell wall consists of three main components:

1. Major protein fractions containing two antigenic distinct proteins termed M and T, which are used to subclassify Group A streptococci. M proteins are necessary for virulence.

2. Group-specific C carbohydrates, which are the basis of the Lancefield immunological reaction.

3. The inner surface of cell wall touches the cytoplasmic membrane, which contains proteins and the lipid bilayer, but no cholesterol or other steroids. The crystal violet staining is a large dye complex that becomes trapped in the thick cross-linked cell wall of the bacteria, resulting in the Gram-positive blue stain, found inside the cells.

Despite the fact that prokaryotes do not engage in sexual union, they undergo genetic exchange, necessary for survival, by simple mutation or by one of the following four ways: transformation, transduction, conjugation, and transposition. All this genetic exchange ensures a genetic diversity that provides the genes that determine antibiotic resistance and the production of exotoxins, enzymes, and other virulent factors.

Some streptococci have also many enzymes that contribute to their pathogenicity, such as:

1. Hawking S, Mlodinow L. *El gran diseño*. 1st ed. 2010: 51.
2. Peterson PY. *Group A and other Streptococci*. In Youmans GP, Paterson PY, Sommers HM, eds. The biology and clinical basis of infectious diseases. Philadelphia: WB Saunders,1975: 172-85.

A. Streptolysin O. The O stands for oxygen-labile, because this enzyme is inactivated by oxygen. It can act on any sterol-containing membrane such as that of red blood cells, leukocytes, platelets, etc. The binding between sulfydryl groups of this bacterial enzyme and the sterols in the red-blood cell membrane changes the three-dimensional arrangement of the membrane lipids. Submicroscopic holes get formed through which haemoglobin escapes into the media, resulting in haemolysis.

B. Streptolysin S. The S stands for oxygen-stabile, and this enzyme is also responsible for the characteristic surface haemolysis.

C. Erythrogenic toxin. This enzyme is found only in a few strains of the Beta–haemolitic group of streptococci and stimulates T cells to release inflammatory cytokines.

D. Other enzymes. These include Streptokinases, Hyalouronidades, DNAses, Anti-C5 peptidases, etc.[3]

The morphology characteristics and the distribution in chains or pairs helped to identify the genus of the Gram-positive cocci found inside tumoral cells as *Streptococcus*. However, the studies of classical bacteriological cultures, MALDI-TOF mass spectrometry, and 16S DNA sequencing failed to recognize any specific species so far.

Streptococcus, a Gram-positive extracellular etiological agent, is one of the most ubiquitous and versatile bacteria pathogen to humans. The bacteria not only colonize the throat, skin, and other organs, but they also adhere to and invade a variety of human epithelial cells. These epithelial cells may provide a nutritional *"rich"* shelter, offering protection to bacteria from components of the host immune system, such as phagocytes and humoral antibodies, and from antibiotics.[4]

An important clinical finding that relates Streptococcus with cancer is the remarkable association between *Streptococcus bovis* infection and colonic cancer. In a study, 50% of people with *Streptococcus bovis* bacteremia had a colonic malignancy.[5]

3. Predisposing factors of cell invasion

The predisposing factors of cell invasion are unknown, but it is believed that they are commonly those that increase the frequency of tumors and endosymbiosis, such as radiations and chemical products exposure, and other additional factors such as aging, alterations of the defenses, cellular integrity, virulence factor of the bacteria, etc.

The worldwide distribution of streptococci could explain the widespread occurrence of malignant neoplasms in all parts of the earth, in many different eukaryotic species and in all organs of their bodies. The change from saprophytism to intracellular parasitism can be due to genomic mutations produced by phages or incomplete viruses, as it happens in Paramecia infected by bright Kappa particles. All genetic or environmental factors produce either an increasing number and virulence of streptococci or an induction of cell membrane lesions and impaired cell defenses against streptococci. Most of the known carcinogens (group I) associated with cancer, especially lung cancer, act on one or both the following pathways: smoking, cannabis abuse, chronic obstructive pulmonary disease, acquired immunodeficiency syndrome (AIDS), and multiple exposures to agents such as ionising radiation, asbestos, silica, nickel, chromium, arsenic, vinyl chloride, bis (chloromethyl) ether, and polycyclic aromatic components.[6]

Streptococci are part of our normal saprophytic bacterial flora and are widely found in the human body, where they live on our skin, nose, oropharynx, mouth, and genitourinary tract.

[3] Peter G, Smith AL. *Group A streptococcal infections of skin and pharynx.* N Engl J Med 1977; 297: 311-7.

[4] Medina E, Rhode M, Chhatwal GS. *Intracellular survival of Streptococcus pyogenes in polimorphonuclear cells results in increased bacterial virulence.* Infect Immun 2003; 71: 5376-80.

[5] Klein RS, Recco RA, Catalano MT, Edberg SC, Carey JI,Steigbigel NH. *Association of Streptococcus bovis with carcinoma of the colon*. N Engl J Med 1977; 297: 800-2.

[6] Brambilla E, Corrin B. In : Gibson GJ, Geddes DM, Costabel U, Sterk PJ, Corrin B, eds. *Respiratory medicine*. Philadelphia: WB Saunders 2003: 1819-27.

4. Route of cell invasion by *Streptococcus*

Cellular membrane restricts penetration of most bacteria and thus, in normal conditions, some bacteria can be found inside lysosomes, but not outside these organelles. The mechanisms of entrance into the cytoplasm may take place through holes in the membrane or, more probably, by increased cell phagocytosis.

Streptococci organisms, specially Group A streptococci, are considered to be highly adhesive extracellular pathogens. However, it has recently been reported that streptococci have the capacity to efficiently invade eukaryotic cells. Interaction of the fibronectins of streptococci on non-phagocytic epithelial cells triggers bacterial internalization. Blocking of adhesins with antibodies or against fibronectin-binding domains prevent streptococci attachment and internalization.[7] Streptococci adhere and invades a great variety of types of cultured human epithelial cells, and several of its adhesive components, known as invasins, promote the internalization of the bacteria.

A number of bacterial species are able to enter host cell through their internalization in phagosomes or endosomes. Following the maturation of these compartments, fusion with lysosomes results in bacterial degradation. However, some bacteria have strategies to avoid the host defense system, which allows the pathogen to escape from endocytic compartments into the cytoplasm (they interfere with lysosomes or their lytic action, or they modify the lysosomes environment).

Streptococci enter human epithelial cells via engulfment by early endosomes; thereafter, endosomes containing streptococci disappear within a few hours, indicating its escape into the cytoplasm. This displacement has been reported to be mediated by Streptolysin O.

But eukaryotic cells have another cellular homeostatic mechanism, which, such as the endocytic pathway, is ubiquitous. It is called "autophagy" and it provides bulk degradation of organelles and cytosolic proteins. During this process, regions of cytoplasm and organelles are first engulfed by double or multiple membrane structures, called autophagosomes. These autophagosomes are formed through the elongation and closure of cap shaped cistern, termed isolation membranes. The trapped material is completely separated from the rest of the cytoplasm and is delivered to lysosomes for hydrolytic degradation. This system promotes degradation and removes damaged organelles such as mitochondrias, periosomes, and even nuclei.

Following the escape from endosomes to cytoplasm, streptococci are found to induce the autophagia machinery through G proteins (Rab5 and Rab7) and become trapped by autophagosomes to later fuse with lysosomes for degradation.

The autophagic machinery seems to be more effective against intracellular streptococci than the endosome-lysosome pathway.[8][9][10]

Despite all that, some streptococci evade also the autophagic degradation, and the pathogenicity is greatly enhanced since penicillin and other antibiotics do not penetrate well inside mammalian cells. These findings support the idea that an intracellular reservoir of streptococci remains viable in a variety of eukaryotic cells such as epithelial cells or macrophages.[11][12]

Streptococci are aerobic germs, but they can live inside cells, becoming facultative anaerobic agents, by modifying their metabolism and adapting themselves to the new ecological niche.

[7] Molinari G, Talay SR, Valentin-Weigand P, Rhode M , Chhatwall GS . *The fibronectin- binding protein of Streptococcus pyogenes Sfbl , is involved in the internalization of Group A streptococci by epithelial cells*. Infect Immun 1997; 65: 1357-63.

[8] Molinari G , Rhode M, Guzman CA, Chhatwall GS. *Two distintc pathways for the invasion of Streptococcus pyogenes in non- phagocitic cells*. Cell Microbiol 2000; 2: 145-54.

[9] Amano A, Nakagawa I, Yoshimori T. *Autophagy in innate immunity against intracellular bacteria*. J Biochem 2006; 140: 161-6.

[10] Sakurai A, Maruyama F, Funao J, Nozawa T, Aikatawa C, Okahashi N, Shintani S, Hamada S, Ooshima T, Nakagawa I. *Specific behaviour of intracellular Streptococcus pyogenes that has undergone autophagic degradation is associated with bacterial streptolysin O and host small G proteins Rab 5 and Rab 7*. J Biol Chem 2010; 285: 22666-75.

[11] Marouni MJ, Barzilai A, Keller N, Rubinstein E, Sela S. *Intracellular survival of persistent Group A streptococci in cultured epithelial cells*. Int J Med Microbiol 2004; 294: 27-33.

[12] Calvinho LF , Oliver SP. *Invasion and persistent of Streptococcus dysgalactiae within bovine mammalian epithelial cells*. J Dairy Sci 1988; 81: 678-86.

In the last step, Streptococcus infects the nuclei of cells which cytoplasm is parasited. The mode of infection by nucleus-specific bacteria is unknown in most of endonucleosymbionts. Information is available only for Holospora species (see Chapter 11). When *H. elegans* and *H. obtusa* approach the micro and macronucleus, membrane projections of the nuclear envelope are generated, which surround the symbiont and after that the bacterium is passed into the nucleus.

5. Effects of streptococci on host nuclei

The effects of streptococci on nuclei are different in resting cells (interphase) or during the mitotic cycle. Let us first describe the effects on nuclei in interphase. Depending on the relative rates of reproduction of streptococci and neoplastic cells, we may find three different situations:

A. The binary fission of streptococci is very fast compared with tumoral divisions, leading quickly to neoplastic cells becoming overcrowded with streptococci. About 10% of the tumoral cells observed within 6 min after surgical removal were dead, and they were overcrowded completely with living cocci. This situation reminds the death of *P aurelia* endosymbiont-bearers placed in axenic culture, due to an excessive reproduction of endosymbionts, whereas endosymbiont-free strains multiply perfectly well in the same medium. The conclusion that unusual environmental conditions can upset the balance between Streptococci and the host, leading in some cases to the death of the neoplastic cell, seems unavoidable. Dead cells appeared with irregular nuclear cavities containing living bacteria, asserting their chromatolytic effect.

B. Steady growth state of streptococci and host cells produces a controlled bacterial accumulation inside nuclei, determining the classical neoplastic aspect of tumors. The most generalized changes induced by streptococci in tumoral cells are the increase of the size of nuclei and cytoplasm and the anomalous shapes of these, mainly due to the storage of cocci inside cells. Streptococci cause pathological nuclei with a great variation of shapes and sizes, which also characterizes the typical aspects of cytological malignancies that allow their diagnosis. Both described above situations can occur simultaneously in different parts of the same neoplasm.

C. The division rate of the cells exceeds the division rate of streptococci, causing a decrease of number of bacteria per cell. The anomalous nuclear and cellular augmentation does not occur if cells can multiply at a rate that matches or exceeds the binary fissions of streptococci. This condition would happen in highly aggressive neoplasms with a fast tumoral growth rate, resulting in malignancies with smaller cell sizes than non-tumoral homologous cells. Our surgical series do not have any case of small-cell carcinoma, because its usual form of clinical presentation is often a widespread disease, and consequently, in most of cases, these tumors are not resected. This peculiar relation between small cell size and fast growing tumor is in line with the biological model observed in paramecia, which can multiply at a high rate: in such cases, the number of symbionts per paramecium is much lower. The reduced number of cocci inside small-cell carcinoma must yet be confirmed.

To sum up, the balance between the reproduction rates of streptococci and the cells can produce three different neoplastic situations:

1. A higher division rate of streptococci causes tumor necrosis.
2. A normal division rate in streptococci and cells results in increased size and anomalous shape of cells.
3. A faster cell division is probably associated with small tumoral cell sizes.

6. Effects on nuclei during mitosis

Streptococci cause an increase in mitosis, which can be observed in smears of malignant cells obtained from tumors taken immediately from surgery and stained with lacto-orcein. About 3% of all malignant cells could be found in a mitotic phase, whereas no division signals could be seen among non-neoplastic cells obtained from normal lung tissue. Fluorochrome staining techniques allow a better study of the tumoral mitotic cycle. About a third of the cells show an average of three potentially new nuclei but, in certain occasions, these values rocket: one may find up to 75% of the cells with new nuclei and some cells may contain up to 30 nuclei (Chapter 8).

As a result, the potential to produce new cells may range from about 20 daughters per 100 neoplastic cells to five times more.

Streptococci use mitosis to pass into daughter cells and are transmitted into the new nuclei. This form is clearly demonstrated on Gram stain and it is called the reproductive form, and has two consequences. Firstly, a morphological effect, in which a variation of cell sizes and shapes can be found. Adult big cells are mixed with many new small cells, which characterizes the pleomorphism seen in malignant tumors. The nuclei of the cells can range from small and hyperchromatic to large and relatively clear showing abnormal shapes. Secondly, given that the diameter of the new cells is close to 7 µm, their dispersion is not easy and these endosymbionts become mostly responsible of local tumor growth. This is shown by the statistical relation between the number of new cells and the size of the neoplasm.

If this model was true, it would also explain the behavior of benign tumors. In these, as the bacterial endosymbionts have only the reproductive form, they induce well-circumscribed local growth, and therefore the surgeon may "shell out" the lesion. This is also the case of certain malignant tumors that show only local invasion, as occurs in solitary bronchoalveolar carcinomas with high resectability and survival rates. This statement is yet to be confirmed because in the observed series there were no benign tumors (inflammatory pseudotumors are neither true tumors nor bronchoalveolar variants from a histopathology point of view).

Taking advantage of cellular division, some streptococci may develop into the infectious form and remain out of nuclei, and they may be released into the extracellular medium. These infectious streptococci are larger than the reproductive form, with a diameter close to 1 µm, as measured with fluorochromes and Gram staining. They are abundant (about 100 per cell) and, given their small size, can spread to long distances and be putatively responsible of the formation of metastasis. In our surgical series, there were three cases of lung metastases from 3 common places of pulmonary metastases: alimentary, genital tracts and kidney, and they showed all cells bearing cocci. The infectious form does not occur in benign tumors and thus, the risk of metastasis would be lower or inexistent.

7. Effects of Streptococci on host cell viability

Streptococci have multiple effects on cell characteristics and their evolution. They modify so much the cell morphology and physiology that the result can be considered a new cell with very different behavior and characteristics compared to the non-cocci-bearer cells, hence their names neoplasm and neoformation. This is an example of evolution towards a new species due to the effect of an endonuclear symbiont.

Streptococci have several metabolic effects that result in changes in growth, metabolic homeostasis, fecundity, stress resistance, and lifespan. The most known and practical effect is the increment of glucose uptake by neoplastic cells, which allows the use of Positron-emission tomography to detect the radioactive isotope 18-F deoxyglucose, an excellent biomarker for the detection, localization, and staging of tumors in patients with cancer.[6]

The improvement of metabolism may lead to an enhancement of the reproductive success and generate exponential neoplastic growth, in a similar way as the fabulous progression of one rye grain in a chess board, according to the known Arabian legend. Tumors weigh 1 g after 30 cell doublings, and after 38 doublings, they rise up to 1 kg, which is a fatal size.[13]

8. Injury effects on non-neoplastic cells

Streptococci, similar to Kappa particles in paramecia, induce the cells' synthesis and release of an impressive amount of chemical substances, which, according to their origin, can be classified as:

A. Tumoral products such as hormones, oncofoetal antigens, enzymes, metabolic products, etc.

B. Chemical substances induced by the tumor, but synthesized by non-malignant cells, such as acute-phase globulins, hydroxyproline, etc.

[13] Geddes DM. *The natural history of lung cancer: a review based on rates of tumoral growth*. Br J Dis Chest 1979; 73: 1-9.

C. Neoplastic antigens, most of them used in clinical practice as biomarkers,[14] despite their lack of specificity.[15][16]

About 15–20% of tumors induce a variety of clinical syndromes that are not related to the direct action of neoplasms, but rather, the consequence of chemical substances production and that may affect any part of the body; these are called paraneoplastic syndromes.[17]

At the onset of their clinical picture about 30% of all bronchopulmonary carcinomas present symptoms of weight loss and low appetite, attributed to the overproduction of cytokines and other mediators.[18][19] Less frequent symptoms are finger clubbing,[20][21] fever, and endocrinology alterations such as hypercalcaemia and low plasmatic sodium levels.[22]

The association of pulmonary thromboembolism with adenocarcinomas is relatively frequent, because of an increase of pro-coagulants factors.[23][24] The list of paraneoplastic syndromes include haematological,[25] neurological,[26] and dermatological alterations.[27] The final outcome is an anatomical and physiological disturbance that modifies body homeostasis and enhances tumoral spread.

9. Benefit to endonuclear symbionts

Attempts to grow symbionts in in-vitro cultures are frequently unsuccessful. The inability to culture bacteria outside their host cells severely hampers their identification and the investigation of symbiotic interactions. Only the advent of molecular biology techniques and the establishment of a phylogenetic system for classification of prokaryotes based on ribosomal RNA have enabled a more detailed characterization of these elusive bacteria. As a matter of fact, streptococci, which are a common and well known bacteria, have remained hidden in tumoral cells from investigators. Streptococci were observed to growing in vitro in six occasions, and only once from a non-tumoral sample (Table 9/1). But usually streptococci grow well on standard culture media and these contradictory results have no clear explanation at the present; perhaps intranuclear strains of streptococci have several essential biosynthetic pathways truncated and, consequently, rely on the input of several metabolites from their host. This probably made the bacteria well adapted for survival in eukaryotes. The nucleus may be considered a favorable habitat since streptococci are provided with nutrients and sheltered from digestion by lysosomal enzymes.

14 Coombes RC, Ellison ML, Neville AM. *Biochemical markers in bronchogenic carcinoma.* Br J Dis Chest 1978; 72: 263-9.

15 Pandha HS, Waxman J. *Tumor markers.* Q J Med 1995; 88: 233-41.

16 Ferrigno D,Buccheri G. *Clinical applications of serum markers for lung cancer.* Respir Med 1995; 89: 587-97.

17 Schnider BI, Manalo A. *Paraneoplastic syndromes. Unusual manifestations of malignant disease.* Dis Mon 1979; 25: 45-96.

18 Chamberlain JS. *Cachexia in cancer-zeroing in on myosin.* N Engl J Med 2004; 351: 2124-5.

19 Theolofides A. *Anorexins, asthenins, and cachectins in cancer.* Am J Med 1986; 81: 696-8.

20 Penson RT, Rudd RM. *Octreotide and hypertrophic pulmonary osteoarthropathy.* Thorax 1979; 52: 297-8

21 Sridhar KS, Lobo CF, Altman RD. *Digital clubbing and lung cancer.* Chest 1998; 14: 1535-7.

22 Rassam JW, Anderson G. *Incidence of paramalignant disorders in bronchogenic carcinoma.* Thorax 1975; 30: 86-91.

23 Ramsay T, Rodgers MB. *Systematic review: Trousseau syndrome revisited; should we screen extensively for cancer in patients with venous thromboembolism?* Ann Int Med 2008; 149: 322-33.

24 Ogino H, Hayashi S, Kawasaki M, Nakanishi M, Hara N. *Association of thrombosis-inducing activity (TIA) with fatal hypercoagulable complications in patients with lung cancer.* Chest 1994; 105: 1683-6.

25 Johnson RA, Roodman GD. *Hematologic manifestations of malignancy.* Dis Mon 1989; 35: 721-68.

26 Posner JB. *Neurologic complications of systemic cancer.* Dis Mon 1978; 25: 1-56.

27 Kurzrock R, Cohen PR. *Cutaneous paraneoplastic syndromes in solid tumours.* Am J Med 1995; 99: 662-71.

Besides these general advantages, streptococci induce multipolar cell divisions and produce supernumerary neoplastic nuclei, which results in a regular distribution of streptococci into the daughter cells (reproductive form). Metabolic changes might be a trigger for the development of the infectious form, in which the cocci are longer than the reproductive form because they grow and stop dividing. This makes their recognition easy.

The infective streptococci are released into the extracellular space, where they avoid phagocytosis by leukocytes and other body defenses by producing several well-known local factors such as biofilm, and thus, they can halt the body inflammatory response, resulting in a silent spread.

Clusters of infective streptococci can behave in two ways, similarly to the Holospora model:

1. Streptococci infect normal cells, where they undergo multiple fissions inside the nucleus and develop new reproductive forms, with the consequent spreading of synchronous or metachronous metastases.[28]

2. Infective streptococci cannot quickly invade any target cell. In this case, the fate of these bacteria is not clear. It seems unlikely that these infective forms are able to divide and grow significantly in intercellular spaces. They are probably antibiotic resistant, which would indicate that they neither divide nor show any significant metabolic activity. In consequence, they can remain as unnoticed commensals in different tissues. The risk of bronchial colonization by potential pathogenic bacteria was seen in patients with lung carcinoma, where streptococci were found in the 13% of resected lung carcinomas.[29]

When local or general conditions change and help these streptococci to invade other cells, including those of a histological type different from the primary tumor, they may become responsible for early and late relapses or a second primary cancer.[30][31]

In each cell division, both the reproductive and the infective forms produce up 30 new streptococci-bearer malignant cells and close to 100 free streptococci, able to reach and infect, theoretically, another 100 normal cells in any place of the body. This fast progression, together with the detrimental effects produced on the metabolism of the host, explain the natural history of malignant neoplasms, characterized by unrestrained growth and dissemination.

[28] Sooral AS. *Staged bilateral lobectomy for synchronous bilateral squamous cell carcinoma of lung.* Thorax 1978; 33: 511-4.

[29] Ionas M, Angrill J, Baldo X, Arancibia F, Gonzalez J, Baner T, Canalis E, Torres A. *Bronchial bacterial colonization in patients with resectable lung carcinoma.* Eur Respir J 2002; 19: 326-32.

[30] Duchateau CJ, Stokkel MPM. *Second primary tumours involving non-small cell lung cancer. Prevalence and its influence on survival.* Chest 2005; 127: 1152-8.

[31] Van Bodegom PC, Wagenaar SS, Corrin B, Baak JPA, Berkel J, Vanderschueren RGJRA. *Second primary cancer. Importance of long term follow up.* Thorax 1989; 44: 788-93.

CHAPTER

13 STREPTOCOCCI: ETIOLOGY OF MALIGNANT TUMORS

1. Introduction

In 1882, Robert Koch described four postulates to establish a causal relationship between an infective agent and a specific disease. Streptococci as the etiological cause of neoplasm fulfill one of the four postulates: streptococci are present in every case of tumor and they can be identified. Intracellular strains of Streptococci do not grow in general media for Gram-positive cocci, nor they cannot be identified by genetic sequencing of 16S DNA, extracted from malignant cells. Until now, it has not been possible to experimentally inoculate streptococci into an animal to reproduce the tumoral disease and to extract the bacteria from the infected animal, which constitute the two remaining Koch's postulates.

There are many illnesses, including infectious diseases that fail to fulfill some of Koch's postulates. In 1965 Bradford Hill proposed new criteria to explore the strength of the causal link between infective agent and illness.[1] [2] The application of Bradford Hills' postulates about the etiological role of streptococci on neoplasm can be described as follows:[3]

1. **Temporal cause-effect relationship.** That means that the cause should obviously precede the effect. This cannot be explored with this type of work, because the putative cause (cocci) and effect (malignancy) are studied at the same moment in time. Although it is absolutely necessary that the cause appears before the effect, the only temporal sequencing is a weak proof.

2. **Strength of the association.** This can be expressed either by a high relative risk or by a high odds ratio, and both statistics are an excellent measure of the link cause-effect. The odds ratio between the presence of intranuclear cocci and the risk of cell malignancy was 134 in the lacto-orcein staining experiments, 93 in those with Gram staining, and indeterminate for those with fluorochrome, showing a strong association ($\chi^2 > 20$, $p = 0.000$). In fact, intranuclear streptococci are a pathognomonic feature of neoplasms, because we do not know this finding in any other pathological situation.

3. **Dose-response.** This means that a variation of the proposed cause corresponds with a change of the effects. When a good correlation exists, this is a strong argument in favor of causality, but not absolutely proof due to the possibility of confusion factors facing a spurious correlation. Cocobacillary counts with lacto-orcein staining had an acceptable dose-response curve when plotted against the risk of cancer.

4. **Reversibility.** If the removal of a suspected etiological factor provokes decreased or disappearance of pathological disturbances, this affirms the causality bond. However, there are also confusion factors that can explain recovery situations. Furthermore, at present, there are no available techniques for the reduction or elimination of streptococci from inside neoplastic cells, and therefore, the possibility to observe the reversibility phenomenon is inexistent.

5. **Consistency.** When there are several observations in separated geographical areas and in different times, and all of them show the same conclusions, this consistence reinforces very much the etiological relationship. Although this research was carried out by a unique working group, cocci-bearer cells intermingled with normal lung cells on lacto-orcein and Gram stainings were recognized by inexpert observers, with just minimum training. Moreover, their rate

[1] Rose G, Barker Djp. *Aetiology of disease-comparison between individuals.* Br Med J 1978; 2: 1558–9.

[2] Rose G, Barker Djp. *Aetiology of disease-selection of controls.* Br Med J 1978; 2: 1616–7.

[3] Fletcher Rh, fletcher Sw, Wagner Eh. *Epidemiología clínica.* 2nd ed. Barcelona, 1989: 207.

of success in recognizing the infected cells cannot be explained by random chance ($\chi^2 > 19$, $p = 0.000$). Gram staining was a clear and more precise technique and the observations of expert and observers with minimum training were very similar. Therefore, the key discovery in this investigation, the presence of intranuclear Gram-positive cocci, is a consistent finding.

6. **Biological plausibility.** This criterion requires that the proposed cause-effect relationship should be consistent with accepted current scientific knowledge. Endonuclear symbionts as the cause of deep changes observed in malignant cells is in agreement with numerous well known biopathological observations in living organisms, recognized by a long list of biologists during the past 150 years. This is a repeated pattern, in which the insertion of a prokaryote into an eukaryote cell [4] [5] modifies the function and morphology of this cell. Biological plausibility is a weakly criterion because it depends naturally on the state of knowledge, but when it is met it reinforces the arguments in favor of the causal agent (discussion Chapters 11, 12).

7. **Specificity.** In essence, this criterion means "one cause - one disease", and it corresponds mainly to infectious diseases and inherited inborn characteristics of metabolism. Massive occurrence of intranuclear streptococci in tumors is, at present, a specific disturbance observed in all types of malignancy, from epithelial to lymphatic or connective neoformations. The present state of knowledge supports the hypothesis that the diversity of neoplasm categories is probably a consequence of the different histological tissues invaded by single cocci genus, rather than to the action of different pathogenic cocci.

   The statistical values found related to the specificity of finding cocci in cancer cells were: 91.8% (95% confidence interval [CI] 86–96%) for lacto-orcein staining, 90.4% (95% CI 80–97%) for Gram staining, and 82% (95% CI 68–90%) for fluorochromes stainings.

   The finding of bacteria in cells from normal lung tissue may be due to several reasons:

   - The bacteria could be inside specific phagocytic cells, such as pulmonary macrophages, due to their cleaning role. Some of *"normal cells"* containing bacteria have macrophages appearance.

   - The sample observed could contain cancer cells, since the sample was obtained from tissue neighboring lung tumors. This hypothesis cannot be proved, because the stainings used here are not appropriate for distinguishing cytological malignancy.

   - Streptococci as cause of tumors open a new view of their pathogenesis. There is not a sudden transition from non-malignant to neoplastic cell, but a gradual onset of the tumoral nest, beginning with progressive bacteria uptake and storage. This means there may be cells with different degree of cocci invasion outside of macroscopic neoplasms that could be seen before their definitively morphological differentiation.

   - A few bacteria can be observed in normal cells, but not always inside of lysosomes.

8. **Analogy.** The causal effect is reinforced if there are other examples similar to the studied phenomenon. This part was commented under biological plausibility. In any case, this is a weak criterion for cause-effect relationship.

The specific characteristics of these streptococci (living inside nuclei, inducing new changes of structures and functions on host cell) are different to all aspects of the known streptococci. Thus, we propose the name *Streptococcus hispaniae* for this new specie.

To sum up, two of the eight Bradford Hills criteria are not actually fulfilled: temporal cause-effect relationship, and the most important one, reversibility. Demonstrating the latter would mean that it would be possible to eliminate streptococci without damage to the host cell, which could imply finding the definitive cure of cancer.

[4] Esteve Jc. *Une population de type <killer> chez Paramecium caudatum (Ehrenberg)*. Protistol 1978; 14: 201–7.

[5] Preer Jr, Preer Lb, Jurand A. *Kappa and other endosymbionts in Paramecium aurelia.* Bacteriol Rev 1974; 38: 113–63.

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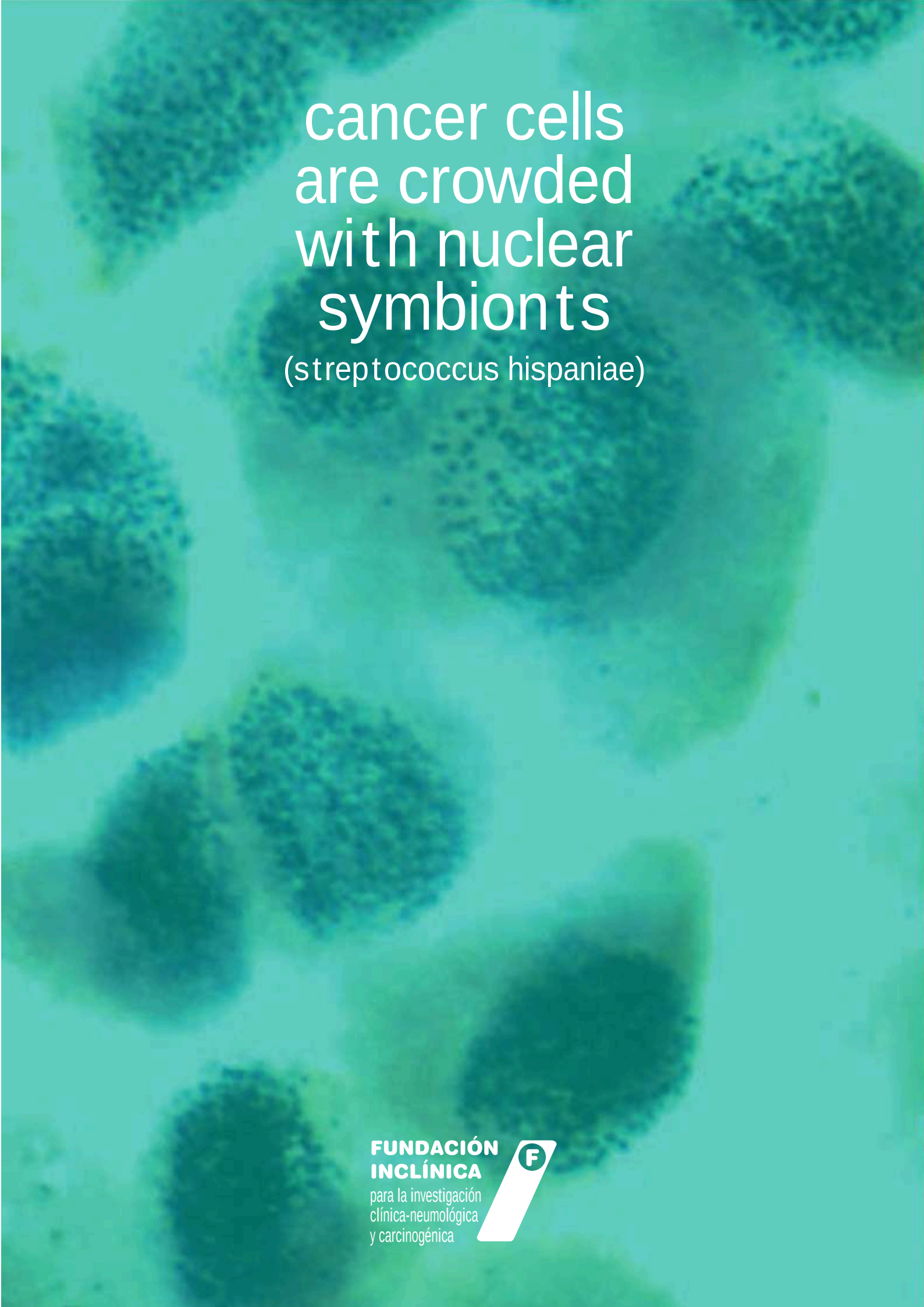

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