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Immunolocalization of cell-wall-deficient forms of *Mycobacterium tuberculosis* complex in sarcoidosis and in sinus histiocytosis of lymph nodes draining carcinoma

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Summary. In sarcoidosis, pleomorphic chromogens (PCs) occur as multivariate pigmented elements within sinusoids of lymph nodes (sinusoidal phase) and as tiny «round bodies» detectable in granulomas (generalized phase). The sinusoidal phase occurs in other conditions as well and characteristically contains yeastlike bodies also known as H-W bodies. To elucidate the antigenic profile of all variant forms, 28 cases of sarcoidosis (series A) and 14 cases of malignancy associated sinus histiocytosis (series B) were studied immunohistochemically with panels of various antibodies, including antimycobacterial MAbs specific for *M. tuberculosis* complex (TB68, TB71), for *M. leprae* (MMP-I-3C3) and for cross-reactive mycobacterial antigens (F24-2-3 and F116-5, the latter recognizing superoxide dismutase). Results for series A indicate that: 1) PCs are cell-wall-deficient (CWD) mycobacterial forms belonging to *M. tuberculosis* complex (over 95%); 2) both phases are antigenically identical parts of the L-cycle; 3) «round bodies» of the «infective» phase have an endolysosomal evolution; 4) uncommon vacuolated forms represent a labile spheroplast stage; 5) the yeastlike bodies are specialized sinusoidal large bodies of unknown function. Results for series B show that in roughly two thirds of cases the pigmented forms are also CWD mycobacteria, have the same immunophenotype as sarcoid PCs in 35.7% of cases, have a much higher incidence of labile vacuolated forms and, finally, that malignancy associated «pseudosarcoid» granulomas do not differ antigenically from genuine sarcoid granulomas. Unlike conventional mycobacteria, PCs do not express cytoskeletal proteins consistently. Their general reactivity for HBcAg raises the possibility of phage interactions being responsible for the L-cycle since it may reflect shared epitopes between unrelated virus entities.

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Introduction

The sinusoids of human lymph nodes may harbor minute intra- and extracellular pigmented elements under diverse conditions, usually in association with sinus histiocytosis. These elements have a native buff to brown pigmentation best seen in unstained sections. They are nearly transparent and do not stain perceptibly with hematoxylin but take up other nuclear dyes avidly and tend to be acid-fast and argyrophilic. Although greatly varying in size and shape the larger elements often assume distinctive yeastlike or spindle-shaped forms with one of a kind smooth double-contoured profiles, which clearly distinguish them from endogenous pigments. They have no accepted name and are here referred to as pleomorphic chromogens (PCs).

Since they were first described in great detail by Hamazaki (1938), pigmented elements of this kind have been investigated repeatedly in lymph nodes of both healthy and sick individuals, particularly in sarcoidosis. They have been described under different names and eponyms (acid-fast spindle-shaped corpuscles, Hamazaki or Hamazaki-Wesenberg (H-W) bodies, yeastlike acid-fast structures, unidentified yellow bodies, yellow-brown bodies, etc). Over the years, they have been variously interpreted as degraded nucleoproteins (Hamazaki, 1938), algal protoplasts (Wesenberg, 1966), lysogenic mutants of tubercle bacilli (Baro and Butt, 1969), giant lysosomes (Doyle et al., 1973; Boutet, 1975), ceroid-ferritin particles (Sieracki and Fisher, 1973), mycobacterial L-forms (Moscovic, 1978) or a form of lipofuscin (Hall and Eusebi, 1978; Ro et al., 1987).

On closer light and electron microscopic scrutiny by one of us (Edward A. Moscovic), the PCs in sarcoidosis

were found to possess unmistakable attributes of viable bacterial cells and were concluded to be tissue forms of cell-wall deficient (CWD) mycobacteria, otherwise known as mycobacterial L-forms. Relevant telltale details have been documented in previous articles (Moscovic, 1978, 1982).

For working and descriptive purposes, the following findings must be re-emphasized: four basic modalities of subcellular organization have been recognized in sarcoid PCs with classical bacterial chromatinic stains (e.g., HCl/Giemsa). These have been arbitrarily designated with Roman numerals based on frequency of occurrence and not as presumptive developmental stages. Types I to III constitute a truly pleomorphic phase occurring strictly within the confines of the sinusoidal network of lymph nodes. Significantly, type IV is not confined to the sinusoids and, in fact, can often be identified within granulomatous lesions regardless of site or organ involved. It occurs in the form of tiny «round bodies» that ultrastructurally appear to be arising from an endolysosomal cycle in epithelioid and giant cells. With the exception of a cursory mention of «small rounded, often double-countoured elements» by Schaumann (1941), this monomorphic generalized phase has, to the best of our knowledge, not been investigated in sarcoidosis previously.

The use of immunohistochemistry has opened a new approach to exploring possible antigenic diversity behind identical morphology and vice-versa. Preliminary testing of sarcoid PCs with commercial polyclonal antimycobacterial antibodies produced inconclusive results that varied with the procedure and chromogen used. Polyclonal antibodies of this kind are broadly cross-reactive and false immunohistochemical positivity cannot be ruled out. The present study has been made possible by the availability of monoclonal antibodies some of which fulfill the long felt need to recognize species specific epitopes of pathogenic mycobacteria. Our primary aim was twofold: first, to probe the immunoprofile of PCs in established cases of sarcoidosis for the presence of mycobacterial antigenic determinants, lysosomal and cytoplasmic proteins, with special focus on their distribution among the various morphologic subtypes, and second, to compare the morphology, distribution and immunophenotype of apparently identical structures occurring in a series of lymph nodes draining carcinoma.

Materials and methods

Selection of cases and tissue processing

Cases for the two test series A and B were collected prospectively and retrospectively between 1981 and 1994 from the surgical pathology files at Harlem Hospital Center, New York. Criteria for inclusion in series A were: established clinical diagnosis of sarcoidosis; representative presence of basic morphologic subtypes among sinusoidal PCs and presence in extranodal or nodal granulomas of «round bodies», numerous and large enough to allow immunohistochemical evaluation by light microscopy; and performance of certain special stains before or concurrent with this study to insure the identity of the chromogenic bodies in keeping with published histomorphologic findings (Carter et al., 1969; Moscovic, 1978, 1982). Criteria for inclusion in series B were: sinus histiocytosis with pigmented elements recognizable in hematoxylin and eosin stained sections of lymph nodes draining malignant tumors with or without metastases; and no known history of sarcoidosis.

In all, 28 cases of sarcoidosis and 14 cases of malignancy associated sinus histiocytosis were selected. Series A comprised formalin-fixed paraffin-embedded samples of lymph nodes with or without granulomas from 20 patients, surgically removed samples of extranodal granulomas in lung (2), spleen (2), breast, skin and tendon from 7 patients and one Zenker-fixed sample of granulomatous lymph nodes obtained from an outside source. The patients were 13 males and 15 females (mean age 40.5, range 16-69 years). Series B consisted of routinely processed lymph nodes draining pulmonary malignancies in 10 cases (6 adenocarcinomas, 2 squamous, one large cell and one small cell carcinoma), mammary carcinoma in two cases and, in one case each, squamous cell carcinoma of the oropharynx and duct cell adenocarcinoma of the pancreas. The patients were 9 males and 5 females (mean age 51.3, range 38-74 years).

For all procedures, five micrometer paraffin sections were mounted on silane-coated glass slides and were deparaffinized and rehydrated in routine fashion. Sections of Zenker-fixed tissue underwent additional treatment with the usual Lugol and sodium thiosulfate solutions for removal of mercury chloride crystals.

Special tinctorial reactions

Prior to immunohistochemical testing, a set of special stains deemed necessary for the histomorphologic characterization of PCs were performed according to previously described techniques on all cases of series B and several recent cases of series A: Ziehl-Neelsen acid-fast (AF), Fontana-Masson silver (FMS), methyl-green pyronin (MGP) (Luna, 1968), direct Schiff (DS) at pH 3-3.5 (for reactive aldehydes according to Carter et al., 1969) and the bacterial chromatinic stains HCl/Giemsa (H/G) or perchloric acid/methyl green (PA/MG) (Moscovic, 1978).

Immunohistochemistry

The primary antibodies used for the localization of other than mycobacterial antigens are listed in Table 1, those used to localize cross-reactive and species specific mycobacterial antigens in Table 2. Because native PC pigmentation may interfere with interpretation, localization was performed in duplicate via standard

peroxidase and alkaline phosphatase based techniques, using commercial peroxidase labeled streptavidin-biotin (LSAB) kits with the chromogen diaminobenzidine (DAB) and alkaline phosphatase labeled streptavidin-biotin (AP-LSAB) kits (DAKO-Corporation) with Fast Red TR (FR) or hexazotized new fuchsin (HNF) detection systems to insure reliable visualization of end-products.

The reagents employed for enzyme digestion were obtained from Sigma Chemical Co, St. Luis, MO, USA. Trypsinization was performed at room temperature (RT) for 20 min with 0.1% trypsin in Tris-hydrochloride buffer, pH 7.6, containing 0.1% calcium chloride. All sections for mycobacterial antigens were processed in duplicate with and without pronase digestion, using a 0.05% pronase E solution in identical buffer for 10 min RT. Sections exposed to enzymes were first chilled and then thoroughly rinsed in distilled water. Those labeled for peroxidase were immersed in a 3% solution of hydrogen peroxide in methanol for 10 min and then repeatedly washed in 0.01M phosphate buffered saline (PBS), pH 7.2. Those destined for AP were first treated with 2.28% periodic acid for 10 min, followed by 0.02% fresh sodium borohydride for 2 min to suppress endogenous alkaline phosphatase activity (Bulman and Heyderman, 1981), and then washed in three changes of PBS.

Incubations with primary antibodies were carried out in humidifiers at 4 °C for 12 to 16 hours overnight, using subsequently the link and labeled streptavidin reagents of the LSAB kits at RT in the usual manner. Color development with DAB was limited to 7 minutes. For AP procedures, two drops of a 30 mg/ml solution of levamisole were added to each 5 ml of FR or HNF substrate and, after filtering the solution, color development was allowed to proceed for 20 min RT. Counterstaining was omitted except for selected sections which were stained with Mayer's hematoxylin (Hx) or with 1% methyl green (MG) in citrate buffer, pH 5.3. Sections developed with DAB and HNF were dehydrated and cleared in routine fashion before coverslipping. Sections treated with FR were mounted with Glycergel (DAKO Corporation), a glycerol-gelatin water-soluble mounting medium.

Known controls were used for cytokeratin (lung), ferritin (fetal liver), hepatitis B virus core antigen (HBcAg) (infected liver) and for three mycobacterial species: *M. tuberculosis* (lung and epicardium from two cases of miliary tuberculosis), *M. leprae* (skin from a case of lepromatous leprosy) and *M. avium/intracellulare* (lymph nodes from two AIDS patients). Internal controls for the remaining antibodies were adequate in most tissues. In negative controls, primary monoclonal antibodies (MAbs) were omitted and primary polyclonal antibodies (PABs) were substituted with commercial non-immune sera (L1802 or L1828, DAKO Corporation).

Table 1. Antibodies against cytoskeletal, lysosomal and miscellaneous antigens.

ANTIGEN	CLONE	DILUTION/ENZYME		SOURCE
Cytokeratin	AE1	1:100		BioGenex
Tubulin	Polyclonal	PD	T	BioGenex
Ferritin	Polyclonal	1:200		BioGenex
Muscle actin	HHF35	1:8000		Enzo Diagnostics, Inc.
Vimentin	V9	1:20		DAKO Corporation
CD 68	KP1	1:60	T	DAKO Corporation
Muramidase (lysozyme)	Polyclonal	PD	T	DAKO Corporation
Desmin	Polyclonal	PD	T	DAKO Corporation
S-100	Polyclonal	PD	T	DAKO Corporation
HBcAg	Polyclonal	PD		DAKO Corporation

HBcAg: hepatitis B virus core antigen; PD: prediluted; T: trypsin.

Results

Histomorphology

The morphology, distribution and staining reactions of the basic subtypes of PCs, as originally defined for sarcoidosis, have been summarized in Table 3. Of the 21 cases with lymph nodes in series A, granulomatous involvement was present in 17 and absent in 4 cases. Intrasinusoidal chromogens were found in all but 5 cases with granulomas in which PC-IV were common. The

Table 2. Polyclonal and monoclonal antibodies against mycobacterial antigens.

ANTIGEN	CLONE/DESIGNATION	DILUTION/ENZYME		SOURCE
**M. bovis*, BCG	Polyclonal	1:800	P	DAKO Corporation
**M. paratuberculosis*	Polyclonal	1:800	P	DAKO Corporation
**M. duvalii*	Polyclonal	1:800	P	DAKO Corporation
**M. tuberculosis*, 16 kD	F24-2-3 (IT-4)	1:1000	P	CDC/WHO, Atlanta GA
M. tuberculosis, 16 kD	TB68 (IT-20)	1:30	P	CDC/WHO
M. tuberculosis, 38 kD	TB71 (IT-23)	1:5	P	CDC/WHO
**M. tuberculosis*, SOD 23 kD	F116-5 (IT-61)	1:5000	P	CDC/WHO
M. leprae, 35 kD	MMP-I-3C3 (mc9247)	1:10000	P	CDC/WHO

*: cross-reactive with other species; BCG: strain Bacillus Calmette-Guérin; P: pronase E; SOD: superoxide dismutase; IT or mc in brackets: CDC/WHO reference numbers.

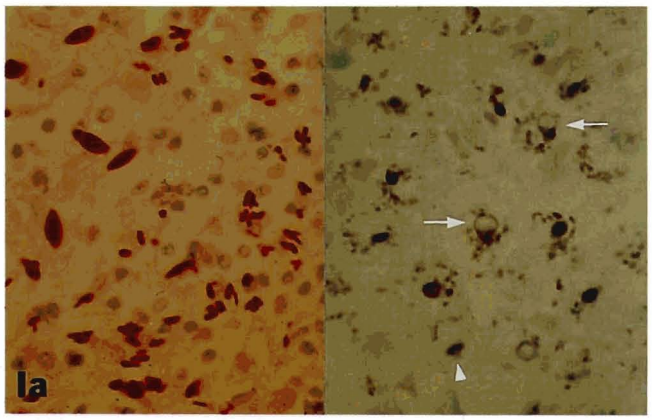
Table 3. Basic morphologic subtypes of pleomorphic chromogens (PCs) in sarcoidosis.

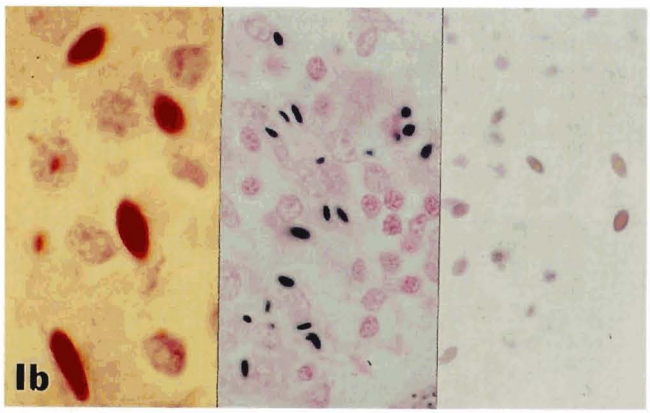
DISTRIBUTION	INTRASINUSOIDAL PHASE (confined to sinusoids of lymph nodes)			GENERALIZED PHASE (including granulomas)
Subtype	PC-I	PC-II	PC-III	PC-IV
Pigment	Buff to brown	Buff to brown	Hypopigmented	Hypo- or non-pigmented
Appearance	Yeast like, often spindle-shaped	Multivariate	Vesicular, often ring-shaped	Round
Profile	Smooth, double-contoured	Indistinct	Smooth	Double-contoured
Size	5-20 µm	up to 6 µm	5-20 µm	up to 3 µm, seldom larger (usually submicroscopic)
"Nucleoid"*	Large, single or multiple	Small, single or multiple	Variable, may be absent	Relatively large, single
Lipoid core**	Present	Absent	May be present	Absent
Staining AF	Variable***	Variable ***	Variable ***	Variable***
FMS	Black	Grey	Grey	Grey
MGP	Pyroninophilic	Pyroninophilic	Pyroninophilic	Pyroninophilic
DS:	Red	Red	Red	Red

*: seen as basophilic core in H/G or PA/MG stains; **: seen as unstained core of "nucleoid" in PA/MG stains, but stains avidly with basic fuchsin and is usually acid-fast even in non-acid-fast forms; ***: case specific, all or none except lipoid cores. AF: Ziehl-Neelsen acid-fast stain; FMS: Fontana-Masson silver technique, used to demonstrate inherent silver-reducing capability without added reducing agent; MGP: methyl green-pyronin procedure gives selective red staining of mammalian RNA, known as "pyroninophilia" (e.g. plasma cells rich in RNA); DS: direct Schiff: application of Schiff reagent at pH 3-3.5 without prior periodic acid treatment stains reactive aldehydes in vivid red.

distinctive yeastlike or-spindle-shaped bodies (PC-I) were found to occur as the only sinusoidal subtype in 6 cases, in association with PC-II in 2 cases and in association with both PC-II and III in 8 cases. Neither PC-II nor PC-III occurred as the sole subtype. «Round bodies», the generalized monomorphic PC-IV, were recognized in 14 nodal and in all 7 extranodal granulomas. They were exceptionally large and numerous in 2 extranodal granulomas and in 3 lymph nodes without detectable sinusoidal forms. On the other hand, they were small and difficult to find in 7 nodal and 3 extranodal granulomas. They were not recognized in 3 cases in which nodal granulomas showed advanced sclerosis although intrasinusoidal forms, mainly PC-I, were present.

The PCs in series B were morphologically





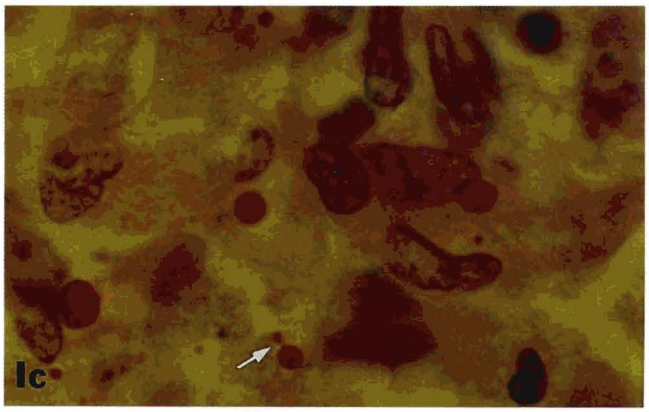


Fig. 1. Staining reactions of chromogens. **a, left.** AF positive. Series B. x 400. **a, right.** AF negative except type I «nucleoids» obscured by counterstain. Two ring forms (arrows) and corpuscle (arrowhead) with typical «explosion vacuole». Series B. x 1,000. **b, left.** Pyroninophilia, MGP. Series A. x 3,000. **b, center.** HCl/Giemsa. Series A x 1,000. **b, right.** Perchloric acid/MG. Series A. x 1,000. **c.** Giant «round bodies» in granuloma, direct Schiff. Note rare sprouting bud (arrow) in one body. Series A. x 1,000

indistinguishable from those in sarcoid lymph nodes. PC-I were present in all cases and often displayed the same kind of granules and «explosion vacuoles». They occurred alone in one case, in association with PC-II in 6 cases and with both PC-II and III in 7 cases. Nodal tumor metastases were seen in 6 cases and «pseudosarcoid» granulomas in 3 cases, in one instance, with recognizable «round bodies» (PC-IV).

Despite a great deal of overlap, the two series showed different trends as regards the frequency of morphologic subtypes and their distribution within sinusoids. In typical sarcoid nodes, PC-I were usually the sole or predominant subtype and tended to occur singly or in loose aggregates in widely separated segments of sinusoids. In malignancy associated sinus histiocytosis, PC-II and III were often more numerous than PC-I and tended to be evenly distributed throughout all sinusoids, sometimes conveying the impression of «pigmented sinus histiocytosis».

Distinctive staining reactions

Results were virtually identical in both series. The exception were two cases of series B, both lymph nodes draining bronchogenic squamous cell carcinoma, in which PCs failed to stain with any procedure other than FMS.

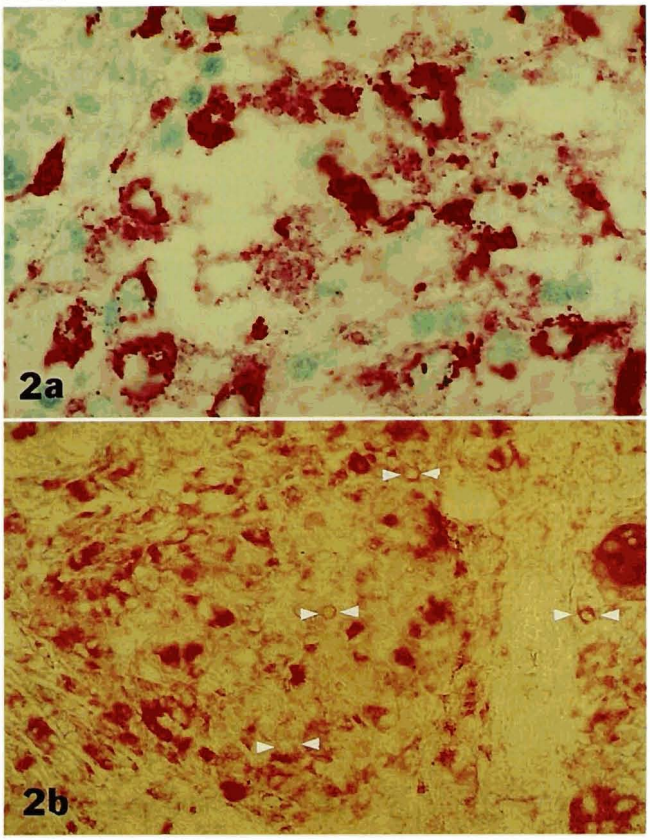
Uniform acid-fastness of all subtypes was seen in 39.2% of series A (7 nodal, 4 extranodal) and in 35.7% of series B (Fig. 1a). Lipoid cores of PC-I were usually acid-fast in negative cases but tended to be obscured by their strong affinity for methylene blue even when lightly counterstained (Fig. 1a). However, if counterstaining was omitted whole corpuscles even in negative cases appeared to be faintly acid-fast, with few exceptions in series B.

Examples of PC pyroninophilia in MGP stains, the selective demonstration of «nucleoids» by the chromatinic H/G stain and the distinctive yellow staining of lipoid cores in PA/MG stains are shown in Fig. 1b. The vivid red coloration of PCs in the DS procedure was most helpful in distinguishing any subtype, but particularly «round bodies», from nuclei or nuclear fragments (Fig. 1c). More details about these staining reactions can be found in Table 3. or in previous reports (Carter et al., 1969; Moscovic, 1978).

Immunostaining

Lysosomal proteins

The PAb against muramidase (lysozyme) and the MAb KP1 against CD68 produced only partly positive results. Small, tightly packed intracellular forms, presumably PC-II, were immunolabeled with both antibodies in 3 cases of series B (Fig. 2a) but showed only rim staining in a few other cases of both series. Large yeastlike bodies (PC-I) were generally non-reactive for lysosomal proteins. In granulomas in which epithelioid and multinucleated giant cells (MGC) were often entirely labeled with both antibodies «round bodies» (PC-IV) could seldom be discerned unless they were exceptionally large or their presence in less reactive cytoplasm could be verified by dark phase contrast. Those that were identified were found to be unlabeled although often surrounded by a narrow rim of immunoreactivity (Fig. 2b).



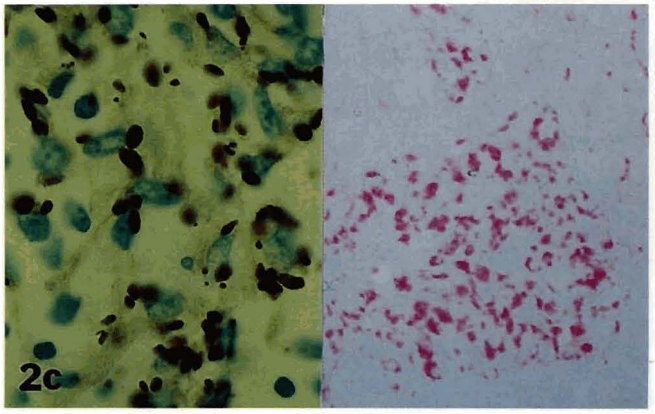


Fig. 2. Immunostaining for lysosomal proteins and for HBcAg. **a.** Small intracellular bodies reactive for CD68 (KP1). Series, B, FR, MG. x 1,000. **b.** Distribution of lysozyme (muramidase) in granuloma. Four large «round bodies» (arrowheads) are negative except for faint rim reactivity. Series A, FR, ncs. x 400. **c.** Examples of staining for HBcAg. Left, series A; DAB, MG, x 1,000; right, series B; FR, ncs. x 200

Cytoskeletal and miscellaneous antigens

The mycobacteria of our three control species in formalin-fixed tissue immunostained for tubulin, desmin and S-100 protein. These reactions were clearly evident in dense aggregates of MAI and leprosy but were too weak to outline individual organisms. Control mycobacteria did not perceptibly immunostain for low molecular weight cytokeratin, vimentin or muscle actin. By comparison, only the Zenker-fixed tissue of series A showed convincing PC reactivity for tubulin and desmin. Although in several cases of either series occasional PCs seemed to stain weakly for tubulin, desmin and in two cases even for vimentin, on the whole, the presence of cytoskeletal proteins could not be conclusively demonstrated in these forms. Immunostaining for ferritin was invariably negative. On the other hand, all subtypes of PCs were to a variable degree immunoreactive for HBcAg in series A and in 9 cases of series B (Fig. 2c). Control mycobacteria did not stain for HBcAg.

Mycobacterial antigens

The frequency with which PCs were labeled for mycobacterial antigenic determinants was clearly related to three factors: type of antibody, prior pronase digestion and morphology of PCs. Not all subtypes reacted equally with the same antibody. The break-down per subtype of maximal reactivity for each antibody after pronase digestion is shown for both series in Table 4. The degree of cross-reactivity or specificity of each antibody for any of the three known control species has also been tabulated.

The greatest differences in reactivity among subtypes were observed with PABs without pronase digestion: most vesicular forms (PC-III) were reactive (Fig. 3a) whereas the distinctive yeastlike bodies (PC-I) were usually not (Fig. 3b). Transitional type III-I forms were often partly reactive in their vesicular components (Fig. 3b). PC-I revealed weak reactivity confined to «nucleoids» in only 4 of 16 cases compared to reactivity in 12 of 16 cases after pronase digestion. «Round bodies» also generally failed to immunostain with PABs. They stained faintly without pronase in just two cases and after pronase digestion in two additional cases.

After pronase digestion, PC-I were often clearly labeled with PABs but patterns of immunostaining varied from full staining of corpuscles to rim staining or staining confined to «nucleoids» (Fig. 3c). Full corpuscular staining was predominant in 8 cases in which other subtypes, mainly PC-III, were also present. Partial patterns of immunostaining, often associated with variable proportions of non-reactive corpuscles, were seen in 4 cases in which PC-I were the predominant subtype. Since this apparent heterogeneity suggested variable resistance to proteolysis two cases with predominantly non-reactive PC-I were classified as positive.

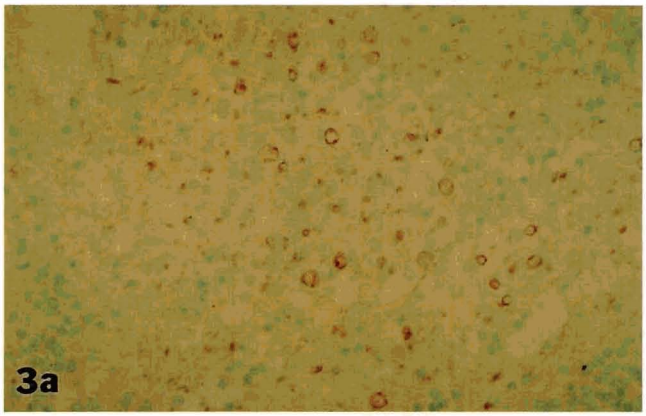
The MAbs F24-2-3 and TB68, which recognize low molecular weight heat-shock proteins specific of *M. tuberculosis* complex, produced positive staining of PCs nearly as often without as with proteolysis. PC-I showed usually full corpuscular staining (Fig. 4a) but rim and «nucleoid» reactivity were also observed, particularly with F24-2-3. In one case, large «round bodies» showed only rim reactivity. Following pronase digestion, two of the MABs (TB68 and F116-5) were associated with significant cross-reactivity with nuclear heterochromatin, often rendering distinction of reactive PCs from host cell nuclei difficult or impossible (Fig. 4b, c). Negative reactions with TB68 were recorded in only one case in which PC-I were the sole subtype and were non-reactive with all other antibodies except «nucleoid» staining with F-24-2-3. The equally species specific MAb TB71 was found to be poorly suited for formalin-fixed tissue and was used on a limited number of cases, with few weakly positive or inconclusive results (Table 4).

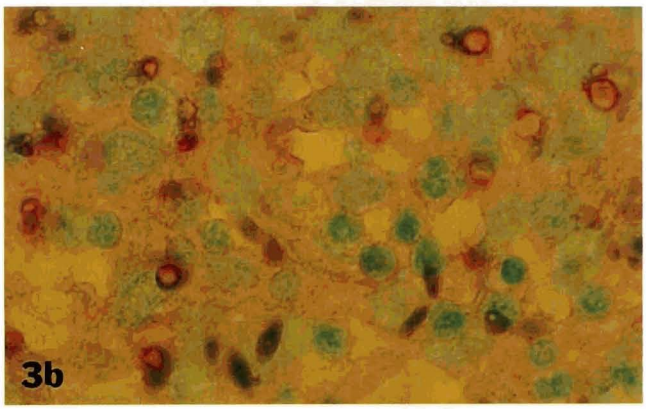
«Round bodies», detected in 21 cases of series A during selection, were identified in only 11 cases by immunohistochemistry. Although they were clearly labeled without pronase digestion with F24-2-3 (Fig. 5a) and TB68 they were often masked by intense granular reactivity induced by the same antibodies in the

Table 4. Results of immunostaining for cross-reactive and species specific mycobacterial antigens in pleomorphic chromogens broken down by subtype in series A and B as compared to known tissue controls.

ANTIBODY		SUBTYPES SERIES A				SUBTYPES SERIES B				KNOWN TISSUE CONTROLS		
		I-II	III	IV	Combined	I-II	III	IV	Combined	TB	MAI	ML
M. bovis, BCG	(P)	12/16	8/8	4/11	16/23	9/14	7/7	0/1	9/14	++	++	++
M. paratuberculosis	(P)	12/16	8/8	3/11	16/23	9/14	7/7	0/1	9/14	++	++	++
M. duvaii	(P)	12/16	8/8	4/11	16/23	9/14	7/7	0/1	9/14	++	++	++
F24-2-3 (IT-4)	(M)	16/16	8/8	10/11	22/23	8/14	7/7	1/1	8/14	++	±	±
TB68 (IT-20)	(M)	15/16	8/8	11/11	22/23	5/14	5/7	1/1	5/14	++	-	-
TB71 (IT-23)*	(M)	2/10	2/8	0/7	2/13	1/9	1/7	0/1	1/9	+	-	-
F116-5 (IT-61)	(M)	3/16	3/8	2/11	5/23	1/14	5/7	0/1	5/14	++	++	++
MMP-I-3C3 (mc9247)	(M)	0/16	0/8	0/11	6/23	0/14	0/7	0/1	0/14	-	±	++

TB: *M. tuberculosis*; MAI: *M. avium-intracellulare*; ML: *M. Leprae*; (P): polyclonal; (M): monoclonal; *: because of its high working concentration this antibody was used on a limited number of cases; ++: moderate; +: weak; ±: negative or equivocal; -: negative immunostaining.





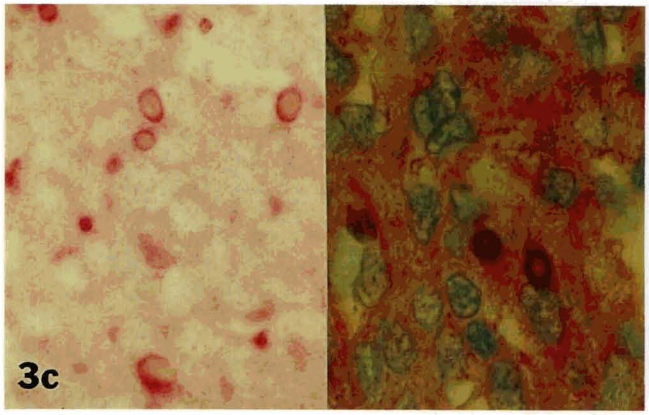
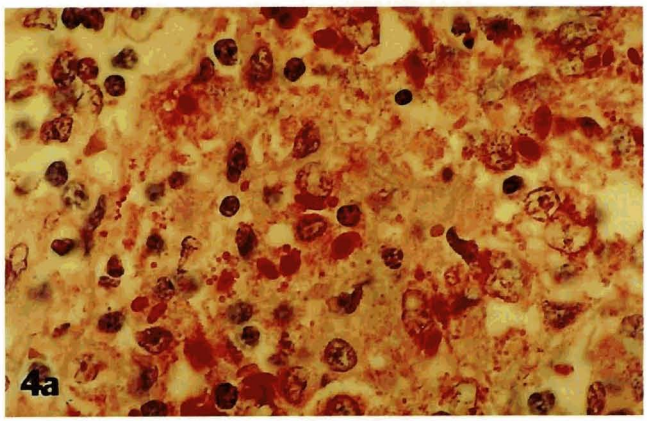


Fig. 3. Immunostaining with antimycobacterial PAbs. **a.** Type III vesicular forms labeled with anti-M-*paratuberculosis*. Series A, FR, MG. x 400. **b.** Close-up view of transitional (III-I) forms partly labeled with anti-*M-duvalii* but non-reactive type I bodies below. FR, MG, x 1,000. **c.** Examples of PCI-staining with anti-*M. bovis* (BCG) After pronase: left, rim staining; series A, FR, ncs, x 1,000; right, staining confined to «nucleoids», series A, FR, MG, x 1,000



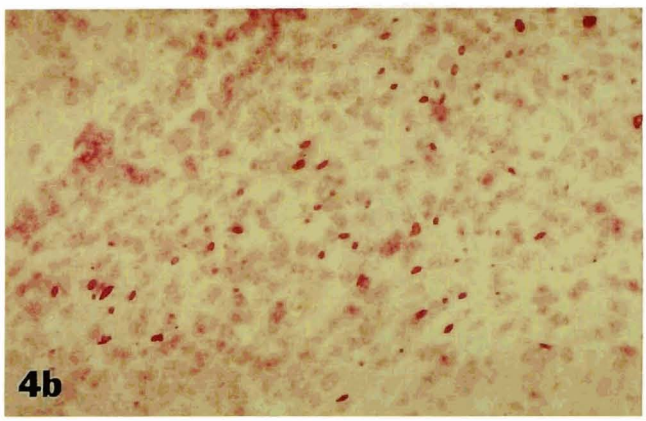
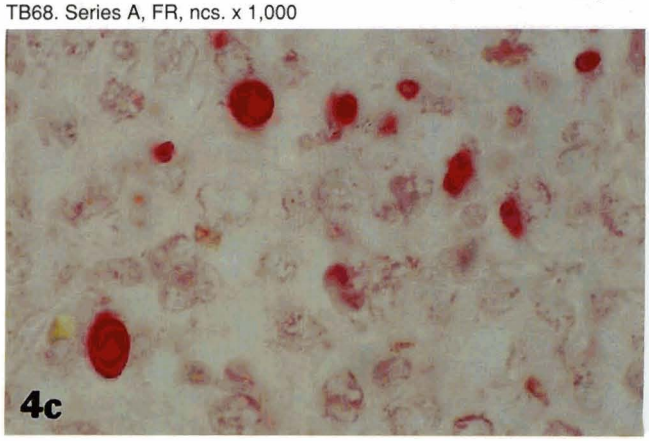
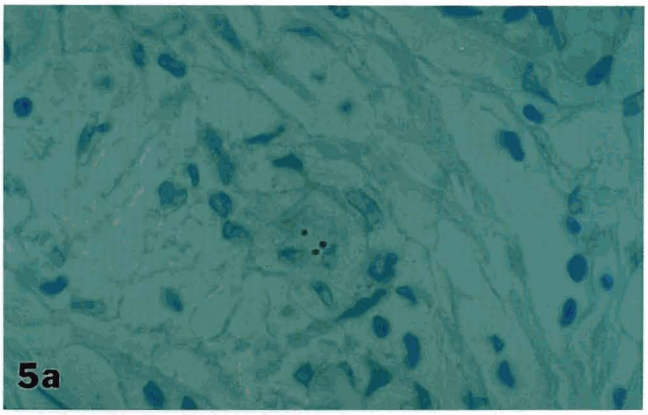
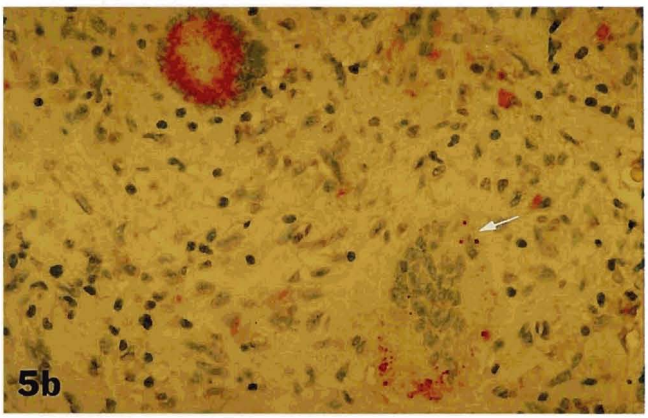


Fig. 4. Immunostaining for species specific mycobacterial antigens after pronase digestion. **a.** F24-2-3 (IT-4) labeling entire corpuscles. Series A. FR, H, x 1,000. **b.** TB68 (IT-20), specific for *M. tuberculosis* complex, labeling PCs intensely but showing substantial cross-reactivity with human DNA. Series A, FR, ncs. x 400. **c.** Close-up view of staining with TB68. Series A, FR, ncs. x 1,000







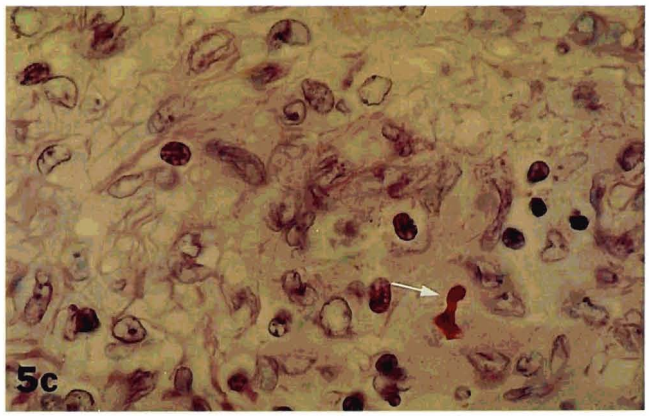
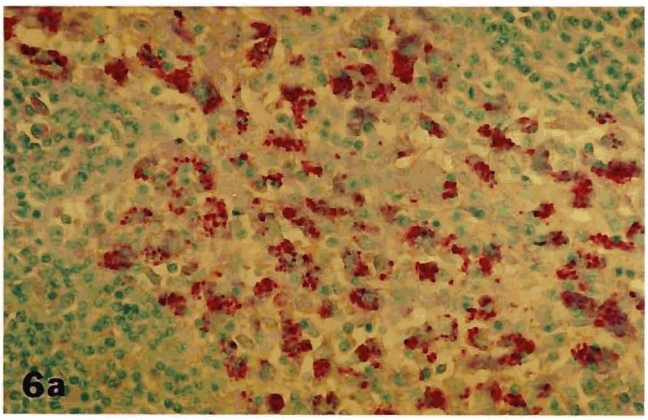


Fig. 5. Immunostaining of «round bodies» in sarcoid granulomas without pronase digestion. **a.** Isolated cluster of 3 «round bodies» in midfield immunolabeled with F24-2-3 (IT-4. DAB, MG. x 1,000. **b.** Strong reactivity for TB68 in cytoplasm of upper MGC precludes recognition of any «round bodies» several of which (arrow) are discernible in lower MGC. FR, Hx. x 400. **c.** Bizarre sprouting «round body» immunolabeled with TB68. HNF, Hx. x 1,000



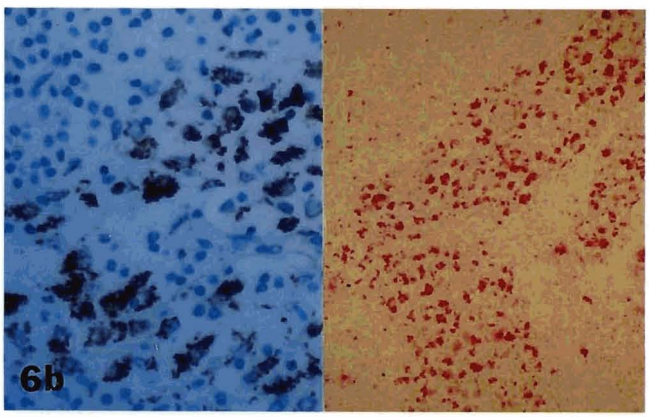
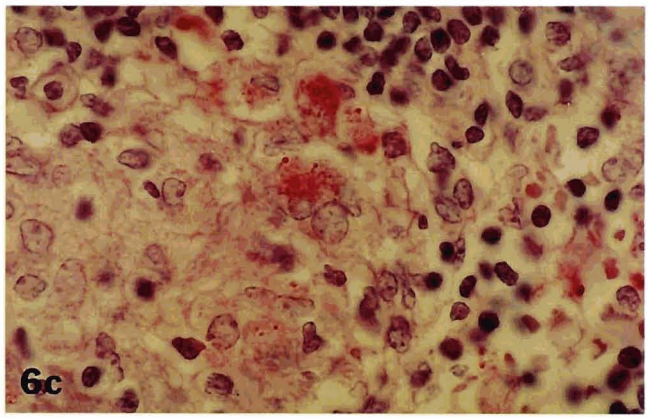


Fig. 6. Examples of immunostaining in «pigmented sinus histiocytosis» (series B). **a.** PAb against *M. paratuberculosis* clearly labels most PCs without pronase digestion (FR, MG, x 400); (**b** left) same case as in (a) staining with TB68 (DAB, MG, x 400) and right, with F116 (IT-61) for superoxide dismutase (FR, ncs, x 200); (c) «pseudosarcoid» granuloma in lymph node draining pulmonary adenocarcinoma revealing few «round bodies» labeled with F24-2-3 (FR,Hx, x 1,000)



cytoplasm of many epithelioid and giant cells (Fig. 5b). The distribution of this cytoplasmic reactivity generally matched that of lysosomal proteins, suggesting abundant amorphous antigenic material in phagolysosomes in addition to uncommon «round bodies». Focal cytoplasmic immunostaining of this kind was noted in at least 3 cases among the 5 cases with no recognizable PCs of any kind. An interesting but rare phenomenon, observed in two cases with exceptionally large and numerous «round bodies», was the presence of bizarre outgrowths from some bodies that were labeled with both MAbs (Fig. 5c).

In lymph nodes draining carcinoma of series B, reactivity with PABs without pronase digestion was quite intense in cases with numerous small intracellular forms (Fig. 6a). However, large PC-I were also usually non-reactive. A total of 9 cases revealed cross-reactivity for mycobacterial antigens after pronase digestion. Of these, 8 cases were reactive with F24-2-3 and 5 cases with TB68 (Fig. 6b, left). Positive staining for superoxide dismutase with MAb 116-5 (Fig. 6b, right) was relatively more common (5/14) than in series A (5/23). The three cases with «pseudosarcoid» granulomas were among the 5 cases with positive TB68 sinusoidal bodies. Significantly, the «round bodies» in the single case in which they were recognized reacted with both F24-2-3 (Fig. 6c) and TB68. In addition, focal reactivity with the same MABs was also seen within granulomas of the other two cases that failed to reveal «round bodies».

Discussion

For some time now, sarcoidosis has been defined as a multisystem granulomatous disease of unknown etiology. This definition, first adopted in 1948 by the Conference on Sarcoidosis in Washington (Ricker and Clark, 1949), has ignored traditional wisdom and experience which viewed the disease as an unusual form of tuberculosis. The widespread acceptance of this new definition as established dogma for the past four decades has effectively curtailed any concerted exploration of the «tuberculous trail» uncovered in the histopathology of the disease by earlier studies.

In fact, the pioneering work of Schaumann and co-workers (Gullberg, 1938; Schaumann, 1941; Hollstrom, 1945) had produced compelling evidence that the disease they called «L.B.» (lymphogranulomatosis benigna) harbored a peculiar lymphotrophic yeastlike variant of the tubercle bacillus. Tangible tissue traces of this agent were described in two forms: as «small rounded, often double-contoured elements» that tended to be acid-fast, and as concentrically laminated «L.B. bodies» found in giant cells and currently known as Schaumann bodies (Schaumann, 1941). Some of the most revealing observations recorded by the Scandinavian investigators were misinterpreted because they antedated generalized knowledge of the bacterial L-phase. However, the phenomenon of reverting L-forms is so distinctive that, in retrospect, the yeastlike forms they depicted as breaking up into typical acid-fast bacilli in glycerine-bouillon media (Gullberg, 1938) are now readily recognizable as mycobacterial L-forms, capable of reverting to conventional bacilli under appropriate conditions. It is clear that if all the work and effort spent on proving a non-mycobacterial etiology of the disease over the past decades had been channeled into pursuing those discoveries the etiology of sarcoidosis may have long been convincingly established and a more rational approach to diagnosis and treatment could by now have been developed.

Nevertheless, sporadic recent reports are reviving interest in the traditional mycobacterial concept of sarcoidosis. CWD mycobacteria have been isolated from sarcoid skin lesions with successful reversion to conventional bacillary forms (Graham et al., 1988). In her extensive studies of CWD bacteria, Mattman (1993) has found that CWD mycobacteria can be readily recovered from the blood of most sarcoid patients in 24 to 48 hours even if the patients' blood is incubated without culture medium. However, recognition of CWD microcolonies requires considerable knowledge and experience, and the species involved cannot be identified without reversion. Modern molecular techniques for the detection of mycobacterial DNA (Bocart et al., 1992; Saboor et al., 1992) or ribosomal RNA (Mitchell et al., 1992) in sarcoid granulomas have also begun to yield some positive results. Unfortunately, these techniques destroy the tissue and are currently unable to localize the target. On the other hand, the immunolocalization of antigenic determinants of *M. tuberculosis* complex in Schaumann bodies, reported most recently (Ang and Moscovic, 1996), proves that for the time being immunohistochemistry is the only reliable method by which the nature of Schaumann's pivotal clues can be explored in situ and verified. Initial results seem to vindicate Schaumann's original conclusions.

The complex nature of mycobacterial protein antigens and the specificity and sensitivity of the antimycobacterial antibodies used in this study have been discussed in a previous report (Ang and Moscovic, 1996). Only properties essential for the interpretation of results are reiterated. Commercial rabbit-raised PAbs cross-react with other mycobacterial species regardless of which species is used for immunization. Since immunization is performed with sonicates containing both soluble and insoluble antigens of the organism these antibodies are presumably capable of binding to the entire spectrum of antigens present in a given species but, in our experience, mainly react with cell-wall-associated antigens which are broadly cross-reactive. The MAbs F24-2-3 and TB68, both members of the low molecular-weight heat-shock protein family, as well as the MAb TB71 which recognizes a phosphate transport «binding protein», have been known for some time to show restricted specificity to *M. tuberculosis* complex (i.e., reacting with *M. tuberculosis*, *M. bovis* BCG and *M. africanum*). MAb F116-5 recognizes the *M. tuberculosis* superoxide dismutase (SOD) which has more than 50% sequence identity with *E. coli*-SOD and

manganese superoxide dismutase (Mn-SOD) of human mitochondria. MMP-I-3C3, the only extraneous MAb of the panel, is specific for a membrane-associated antigen of *M. leprae*. Data available on all of these MAbs have been compiled in a recent review by Young et al. (1992).

Although the WHO MAb bank at CDC recently reclassified F24-2-3 as cross-reactive with other species the cross-reactions we observed with this antibody in our controls were negligible, as were those noted with MMP-I-3C3. Except for TB71, the MAbs of this panel worked well on formalin-fixed tissue without pronase digestion. As regards the paradoxical nuclear immunostaining by TB68 and F116-5, this was seen only after pronase digestion and can be ascribed to shared antigenicity between antimycobacterial MAbs and human DNA which has been brought to light by ELISA previously and has been suggested to play a role in the genesis of autoimmunity (Shoenfeld et al., 1986).

When our results are analyzed, it is clear that pleomorphic chromogens are CWD mycobacterial forms which in sarcoidosis, and in at least one third of cases with malignancy associated sinus histiocytosis, belong to *M. tuberculosis* complex. Even though expression of mycobacterial antigens varied among the four basic subtypes in terms of staining intensity and distribution of immunoreactivity, all subtypes were found to be antigenically part of the same entity. The «round bodies» could, for the first time, be also immunohistochemically linked to the sinusoidal forms as part of the same CWD or L-cycle. This undoubtedly most important finding of the present study confirms the existence in sarcoidosis of a generalized «infective» phase of CWD mycobacteria that can be demonstrated within granulomas by routine light optic means.

«Round bodies» were recognized by one of us more than two decades ago. In sarcoid granulomas from over 500 patients screened over the years, they were detected in roughly 40% of cases, with an incidence ranging from less than 10% in needle biopsies to 100% in large specimens such as lungs or spleens. We have since come to regard them as an important hallmark of the sarcoid granuloma although they are not pathognomonic and may also be found in tuberculous granulomas, particularly in recently treated patients. They also occur in the sinusoids where they have been identified within leukocytes by electron microscopy (Moscovic, 1978).

In the sarcoid series, «round bodies» were identified in eleven cases immunohistochemically. They generally lacked cell-wall-associated antigens but in all cases expressed immunoreactivity for *M. tuberculosis* complex specific antigens. Rim staining for lysosomal proteins supports their endolysosomal evolution which has been suggested ultrastructurally by the presence of two separate unit membranes. Their fine structure resembles that of elementary corpuscles, the reproductive units of the L-cycle (Dienes, 1968), from which they may well evolve.

Given the known capacity of ordinary parasitic mycobacteria to survive and to even thrive within phagolysosomes (Brown et al., 1969; D'Arcy Hart et al., 1972), it may well be expected that the CWD counterpart of pathogenic species should be able to sporadically grow from submicroscopic to a visible size. They exceptionally achieve a bewildering size, indicative of rapid growth. As a result, «round bodies» are not always round and may show irregular protrusions or even rare budding. On the other hand, not only old sclerotic lesions but also active lesions may fail to reveal «round bodies» in the visible size range.

Among the intrasinusoidal forms of both series, the exquisite reactivity of bubble and ring forms (PC-III) with both PAbs and MAbs, including expression of SOD, sets these forms apart from the other subtypes as probable spheroplasts, that is, CWD forms retaining some components of cell walls. Spheroplasts may be viewed as an intermediate labile stage between a stabilized L-phase entirely free of cell walls and conventional organisms with rigid cell walls. Large vacuolated bodies were recognized early in the study of the L-cycle (Dienes and Edsall, 1937) and are now known to be characteristic of L-colonies. Their unusually high incidence in series A (38%) is the result of selection. They are usually uncommon in sarcoid lymph nodes with PCs (incidence below 10%). Their presence in 50% of malignancy associated sinus histiocytosis, not predetermined by selection, would seem to indicate a much higher incidence of labile CWD stages in those conditions than in sarcoid lymph nodes. By the same token, the small intracellular forms in series B, which expressed lysosomal as well as cell-wall-associated mycobacterial antigens, may be phagocytized spheroplasts.

In those cases in which all three subtypes of sinusoidal chromogens are present, there is evidence that type I corpuscles may evolve from the other two subtypes. Because these subtypes, whether intra- or extracellular, are strictly confined to sinusoids and appear incapable of developing elsewhere it is probable that the distinctive yeastlike bodies are specialized forms rather than involuting forms of ebbing viability. In terms of energy stored, they seem to fulfill requirements for a reproductive role (Moscovic, 1982), such as maintaining the L-cycle by release of elementary bodies under propitious conditions, as postulated for large bodies by Dienes (1968). Whether the sinusoidal phase is instrumental in maintaining the generalized phase or vice versa is presently not known.

Paradoxically, the largest and most conspicuous type I corpuscles show most variable immunostaining, often evident in the same section. They are the only subtype with frequent compartmentalized reactivity for both cross-reactive and specific mycobacterial antigens which indicates a special role of the lipoid cores within «nucleoids». Full corpuscular staining, most often seen with F24-2-3, indicates a high content and fairly uniform distribution of low molecular-weight heat-shock proteins although those detected by TB68 are sometimes also confined to «nucleoids». Unlike small intracellular forms, the large yeastlike bodies are not associated with lysosomal reactivity and are unlikely to represent giant

lysosomes. Some of the small intracellular forms may well be endolysosomal or endophagosomal and may not be viable. Electron microscopy is least likely to distinguish viable from non-viable contents of lysosomes. Evidence that some type I corpuscles develop from extracellular large vacuolated forms and lack lysosomal proteins seems to refute previous ultrastructural interpretations of the yeastlike bodies as giant lysosomes (Doyle et al., 1973).

Considering the variable staining patterns of PCs, it is clear that large solid corpuscles are particularly resistent to proteolysis and impregnation by antibodies, possibly due to inseparable lipid-protein binding or to the presence of some extraneous factors such as prophage or maturing phage. The PC reactivity for HBcAg observed in both series also suggests a phage since it may involve shared epitopes between two viral entities however unrelated. This reaction was not observed among control mycobacteria. The inclusion of this antibody in the panel was prompted by results from preliminary testing of sarcoid PCs for common viral antigens (herpes virus type I and II, CMV, EBV and hepatitis B virus surface and core) which indicated positive staining only for HBcAg.

Positive staining of conventional mycobacteria for cytoskeletal filament proteins has been described and discussed by others (Umlas et al., 1991; Kahn and Thorner, 1992). Our controls immunostained clearly for tubulin and desmin but not for low molecular-weight cytokeratin, vimentin or muscle actin. Results from both series A and B suggest that these proteins may be present in PCs in trace amounts but cannot be consistently demonstrated.

Sinus histiocytosis is a well recognized reaction pattern in lymph nodes draining carcinoma. It is common knowledge that often a lymph node draining a malignant tumor will show both metastases and sarcoid granulomas adjacent to one another or only granulomas. While the coexistence of generalized sarcoidosis and malignancy is rarely documented (Ellman and Hanson, 1958; Sakula, 1963) the significance of localized sarcoid lesions seen often in association with malignant neoplasms has been a matter of speculation (Gherardi, 1950; Gregorie et al., 1962). In a brief communication, Epners (1975) called attention to the frequent co-existence of photochromogenic mycobacteria and bronchogenic carcinoma but the granulomas associated with carcinoma generally lack acid-fast bacilli when examined histologically. The occurrence of pigmented elements in the sinusoids has to the best of our knowledge not been investigated in this context.

The observation that all three cases with «pseudosarcoid» granulomas in series B revealed both PC and cytoplasmic reactivity with TB68, in one case with demonstrable «round bodies», indicates that these are genuine sarcoid granulomas that may be caused by the same mechanism of pathogenesis.

Three of the cases in series B were tested at another institution with a newly introduced PCR method for the identification of atypical mycobacterial species in paraffin sections (Cook et al., 1994). The result: positive for atypical mycobacteria, identical pattern identified for each (possible saprophytic contaminants). However, the species could not be identified. Since all three cases reacted positively for *M. tuberculosis* complex in our examinations the failure of PCR to identify the species again raises the possibility of lysogeny. The insertion of phage in the mycobacterial genome, with its site and sequence unknown, may for the time being render PCR ineffective for the species identification of lysogenic mutants.

In conclusion, the findings in this study demonstrate that pleomorphic chromogens of all morphologic subtypes in both nodal sinusoids and granulomatous lesions are cell-wall-deficient mycobacterial forms which in most cases of sarcoidosis (95.6%) express specific reactivity for *M. tuberculosis* complex. We call attention to the tissue forms of the generalized monomorphic phase, «round bodies», as an important hallmark of the sarcoid granuloma and to their endolysosomal evolution.

It is important to acknowledge that in most cases of malignancy associated sinus histiocytosis the pigmented bodies have also a mycobacterial parentage (64.7%), including *M. tuberculosis* complex (35.7%), but may represent CWD forms of other bacterial genera in the rest of cases (35.7%). In our cases, associated «pseudosarcoid» granulomas were present in three cases and were immunohistochemically indistinguishable from genuine sarcoid lesions.

Our findings uphold the traditional view that sarcoidosis is an unusual variant of tuberculosis and provide tangible evidence for the mycobacterial pathogenesis of the disease. We emphasize the fact that cell-wall-deficient rather than classical or anonymous mycobacteria are involved. At the same time, it is clear that only further studies can answer the many questions raised. What is the species or strain of *M. tuberculosis* complex involved? Or are there more than one involved? What factor or factors cause the L-cycle? Is lysogeny involved and, if so, which of the many types of mycobacteriophages is or are responsible? Finally, what is the role played by the pigmented sinusoidal bodies, especially the distinctive yeastlike bodies, which are never found in the lesions but are often found in lymph nodes without any apparent relation to the disease process? Do they represent a dead-end shunt of involuting forms or are these hibernating forms capable of reproduction under favorable conditions?

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