The Structure of Mononuclear Phagocytes Differentiating *In Vivo*

II. The Effect of Mycobacterium tuberculosis

Dolph O. Adams, MD, PhD

The development and resolution of granulomas induced by *Mycobacterium tuberculosis* were sequentially traced by correlated light and electron microscopy. The scattered, immature monocytes initially composing the lesions evolved by orderly steps into coalescent, well-developed macrophages and ultimately into swirling nests of highly complex epithelioid cells. These ultrastructural changes represent differentiation *in vivo* of the mononuclear phagocytes. The number of mycobacteria present then waned markedly, and the epithelioid granulomas developed into foreign body granulomas and finally into simple chronic inflammation. Concomitantly, the epithelioid cells evolved into macrophages and ultimately into immature, monocyte-like forms. These observations suggest that the development of a granuloma represents differentiation *in vivo* of the constituent mononuclear phagocytes in response to an evoking stimulus. From comparisons with previous studies, mononuclear differentiation *in vivo* appears to have a fixed pattern and a markedly alterable pace. The observations also suggest a previously undescribed fate for mononuclear phagocytes in developing granulomas. As the granuloma-evoking agent is destroyed, the highly differentiated mononuclear phagocytes change into less mature forms. (Am J Pathol 80:101–116, 1975)

THE WIDELY DISTRIBUTED host system of mononuclear phagocytes, which comprises monocytes, macrophages, and their specialized progeny, plays important roles in the host's normal economy and defenses against intruders.[1,2] Because these cells leave the marrow in an immature form, their functional capacity relates directly to their state of differentiation.[2,3] Although the differentiation of these cells has been studied mostly *in vitro*,[1,5] recent studies from this laboratory have directly demonstrated their differentiation *in vivo*.[6] The development of epithelioid granulomas induced by bacillus Calmette-Guerin (BCG) was marked by the differentiation of their constituent mononuclear phagocytes. Monocytes differentiated in an orderly fashion into macrophages and ultimately into extremely well-differentiated forms, which we recognize histologically as epithelioid cells.[6] This suggests that

From the Department of Pathology, Duke University Medical Center, Durham, and the Department of Pathology, the University of North Carolina at Chapel Hill, School of Medicine, Chapel Hill, North Carolina.

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Address reprint requests to Dr. Dolph O. Adams, Department of Pathology, Box 3712, Duke University Medical Center, Durham, NC 27710.

the development of a granuloma represents differentiation *in vivo* of its constituent mononuclear phagocytes.

The general validity and applicability of this idea remain to be established because differentiation has yet to be demonstrated in other models of granulomatous inflammation.[4,7] If differentiation were to occur in such models, it might well be dissimilar to that observed in the BCG model, where full differentiation was slow. In a rapidly developing model of granuloma formation, differentiation might evolve by other means altogether or might proceed to a different extent. Alternatively, the orderly pattern of differentiation previously observed might be altered by omission or asymmetrical expansion of various steps.

The means by which granulomas resolve is incompletely understood.[4,7] In particular, the ultimate end of individual mononuclear phagocytes is not clear. Evidence to date suggests that these cells persist for some time and then either die or divide into new, immature forms.[8-12] The fate of these cells has not been studied in terms of differentiation.

For these reasons, a model of rapidly evolving granulomatous inflammation was studied from inception to resolution for the presence and extent of mononuclear differentiation. Differentiation was found to occur but to differ significantly from that seen in the BCG model. Of particular interest, the granulomas resolved by a heretofore undescribed mechanism.

Materials and Methods

Technique for Fluorescent Staining of Mycobacteria

Sections were stained with auramine O after Lempert to detect mycobacteria.[13]

Animals, Animal Techniques, and Preparation of Tissues

These techniques have been previously described.[6] Briefly, guinea pigs, which were used in all experiments, were lightly anesthesized. The granulomas to be sampled were surgically removed and fixed in either Helly's fluid or buffered glutaraldehyde (osmium postfixation). The tissues were then embedded in either paraffin or Epon, sectioned, and examined.

Bacteria

Cultures of *Mycobacterium tuberculosis*, strain H37 RV—a virulent strain of mycobacteria kindly supplied by Dr. H. M. Vandiviere (Gravely Sanatorium, Chapel Hill, N.C.)—were grown on Lowenstein-Jensen medium for 6 to 8 weeks. Bacteria were then scraped from the culture plates and suspended in Dubos' medium. The suspension was ground in a mortar and particles allowed to settle. The resultant supernatant was irradiated for 17 hours with ultraviolet light and then diluted to a final concentration of

100 million (10^8) organisms per cubic centimeter. The final suspension was recultured to exclude viability.

Experimental Design

The guinea pigs were divided into 13 groups of 4 animals each. On Day 0, 3 of the animals in each group received five intracutaneous injections of 10^7 killed *M. tuberculosis* suspended in Dubos' broth. The injection sites were regularly spaced along the right flanks of the animals; 10^7 mycobacteria were injected at each site. Five sites were used to insure adequate numbers of reactions for study. The fourth animal received five similar injections of broth alone and served as a control. From 6 hours to 21 days later, animals were killed, and samples taken for light and electron microscopy.

Although previous studies with BCG had been conducted in fat pads, an intradermal site was chosen because preliminary studies demonstrated that the injection of up to 2×10^9 killed mycobacteria of the virulent strain into fat pads did not produce granulomas large enough to be detected easily.

Classification of Mononuclear Phagocytes

Mononuclear phagocytes in all of the lesions were classified as either monocytes, immature macrophages, mature macrophages, immature epithelioid cells or mature epithelioid cells by the histologic and ultrastructural criteria previously described.[6]

Results

Histologic and Ultrastructural Findings

Six and Twelve Hours

At 6 hours, the reaction was essentially acute inflammation. A light infiltrate of neutrophils and monocytes—small round cells with dark central nuclei and scanty cytoplasm—filled the tissues (Figure 1A). Controls had a similar but less intense response.

Electron microscopy confirmed the mononuclears as typical monocytes.[4,14] These cells each had a small, centrally located nucleus bearing densely clumped heterochromatin, an inconspicuous nucleolus, and a small rim of cytoplasm. The few organelles were sparse round mitochondria, clumped ribosomes, very occasional profiles of smooth endoplasmic reticulum, very rare lysosomes and occasional pinocytotic vesicles.

By 12 hours, a greater number of monocytes, both absolutely and relatively, were present. Occasional mononuclear phagocytes had a monocytic nucleus and enlarged cytoplasm. Control samples had a waning, acute inflammatory response.

One Day

The lesions consisted of a few exuded neutrophils, many monocytes, and occasional immature macrophages. The immature macrophages had central round vesicular nuclei and prominent, ample eosinophilic cytoplasm and distinct cytoplasmic borders (Figure 1B). Control samples had inconspicuous inflammation.

Ultrastructurally, characteristic immature macrophages were present.[6] The nucleus was slightly enlarged and eccentrically located. Peripheral clumping of heterochromatin was evident, and more euchromatin was noted centrally. Nucleoli were more prominent, and the amount of cytoplasm was increased. Profiles of smooth endoplasmic reticulum and Golgi apparatus were visible, as were numbers of free ribosomes, lysosomes, and mitochondria. Also present were activated tissue macrophages, which were occasionally arranged in small aggregates.

Two Days

The lesions consisted of scattered clumps of immature macrophages (Figure 1C). Controls sample had almost reverted to normal.

Three Days

Early but definite granulomas were seen; a granuloma, by the definition employed in this laboratory, is a group of organized or coalescent macrophages (see "Discussion"). The loose nests of macrophages seen before had coalesced to form compact clumps (Figure 1D). The constituent mature macrophages had large eccentric nuclei, open vesicular chromatin, and large prominent eosinophilic nucleoli which were often multiple. The cytoplasm was enlarged and amphophilic or basophilic. Cytoplasmic borders were somewhat indistinct, so that the macrophages blended with one another. Control samples at this and all later stages had no visible changes.

The ultrastructure of the mature macrophages resembled that previously described.[6] The cells extended pseudopods toward and closely approximated one another; nuclei were large and eccentric. Most heterochromatin was marginated against the nuclear membrane; euchromatin was very plentiful. Nucleoli were very large and compact. The cytoplasm was filled with numerous, small, budding lysosomes; free and aggregated ribosomes; Golgi profiles; mitochondria; and plentiful strands of both smooth and rough endoplasmic reticulum. The mature

macrophages had irregular outlines and many branching pseudopods which barely touched those of other macrophages.

Five Days

Large granulomas composed of solid sheets of mature macrophages and immature epithelioid cells were seen (Figure 2A). The immature epithelioid cells were large phagocytes having very large, oval or reniform, open nuclei. Chromatin was very fine, vesicular, and delicately marginated; nucleoli were small and slightly eosinophilic. The cytoplasm was very pale, eosinophilic, and finely granular; cytoplasmic borders were indistinct. Definite mature epithelioid cells were not seen. In areas, macrophages were beginning to form giant cells.

The fine structure of immature epithelioid cells was as previously detailed.[6] The cells closely touched one another and had many pseudopods which were interwoven and intertwined. The eccentric nuclei were larger and had more euchromatin than those of macrophages. The cytoplasm was very complex and was filled with numerous organelles; pinocytotic vesicles were present.

Seven Days

Granulomas were large, solid, compact, coalescent, and composed of immature epithelioid cells and occasional mature epithelioid cells (Figure 2B). Small Langhans's giant cells were seen.

Electron microscopy of the epithelioid cells, mature and immature, revealed that they closely approximated one another. The multiple cytoplasmic pseudopods closely intertwined with those of epithelioid cells, but no tight junctions or desmosomes were present, even in areas of closest contact. The mature epithelioid cells were large oval macrophages closely packed together. They had oval or reinform, eccentric nuclei and resembled the mature epithelioid cells previously described.[6] Their nuclei had scant, marginated heterochromatin and much euchromatin. Nucleoli were prominent and were often activated. The complex cytoplasm possessed much lamellated rough and smooth endoplasmic reticulum. Lysosomes, ribosomes, polysomes, and Golgi profiles were very plentiful. Phagosomes and pinosomes were prominent.

Nine Days

The well-developed granulomas were composed of mature epithelioid cells which swirled together to form typical tubercles. Numerous Langhans's giant cells were seen.

Eleven Days

The granulomas were beginning to resolve, as typified by a less cellular appearance. They were surrounded by granulation tissue infiltrated by lymphocytes and plasma cells. Fewer epithelioid cells were seen. Those present were smaller and had less cytoplasm and smaller, darker, more centrally located nuclei. Cytoplasmic borders were becoming visible. Numerous mature macrophages were noted in and around the granulomas.

Thirteen Days

The granulomas, clearly resolving by this stage, were consistently smaller (Figure 2C). The component macrophages were not confluent. Composed of loose sheets of mature macrophages, the lesions had the appearance of foreign body granulomas. No epithelioid cells, immature or mature, were present. Foreign body giant cells were not seen.

Fifteen Days

Lesions at this stage were no longer granulomas but loose sheets of immature macrophages and monocytes, and were surrounded by granulation tissue, lymphocytes, and plasma cells (Figure 2D).

Eighteen and Twenty-One Days

Lesions were now small, focal collections of monocytes, lymphocytes, plasma cells, and rare immature macrophages.

Fluorescent Antibody Studies

Examination of auramine-O-stained material by fluorescent illumination revealed that bacilli were in large clumps in the interstitium of the loose, edematous dermis at 6 hours. Occasional organisms were phagocytosed. By 12 hours, most of the bacilli were phagocytosed and were in large clumps of 10 to 20 organisms; several hundred mycobacteria were seen per high-powered field. This appearance persisted through 3 days (Figure 3A).

At 5 days, the phagocytosed bacilli were broken into small clumps, so that individual bacilli were discernible. Most of the mycobacteria were within the cytoplasm of immature or mature epithelioid cells. This general appearance continued through 9 days (Figure 3B). During this time, the large groups of mycobacteria were dispersed into small groups and a moderate diminution in bacilli per high-powered field occurred.

On the thirteenth day, the number of bacilli was dramatically changed (Figure 3C). Bacilli had become very sparse; only 4 to 5 could be dis-

cerned per high-powered field. All of the bacilli were within the cytoplasm of macrophages in the granulomas; no bacilli were seen in areas of loose inflammation. On the 18th day, only very occasional bacilli were seen. Some high-powered fields had no bacilli and others 2 to 5 (Figure 3D). By the 21st day, no bacilli could be found, even after extensive searching.

Discussion

The development of epithelioid granulomas in a slowly evolving model has been shown to represent differentiation *in vivo* of the constituent mononuclear phagocytes.[6] The present study traced the development of a rapidly evolving model evoked by killed *M. tuberculosis* in order to compare a rapid response with a slow one. In the rapid model, monocytes—small, ultrastructurally simple cells that initially composed the lesions—evolved into mature macrophages and ultimately into much larger, ultrastructurally complex epithelioid cells. These changes represent differentiation *in vivo* of the monocytes because they closely resemble those of mononuclear differentiation *in vitro,* are characteristic of differentiating cells in general and differentiating hemic cells in particular, and result in obvious maturation of the phagocytes.[6] The present observations of a rapidly evolving granuloma model thus confirm the conclusion, previously drawn from observation of the slow model, that the development of an epithelioid granuloma represents differentiation *in vivo* of the constituent mononuclear phagocytes. The observations thus support the general validity of this concept of granulomatous inflammation.

The term *differentiation* is employed to stress the fundamental similarity between these *in vivo* observations and these *in vitro* observations of Cohn and others.[2] However, this term may not express most appropriately the nature of the changes undergone by mononuclear phagocytes after stimulation, and many authors consequently employ the less committal word *maturation.*[5] Maturation, too, may not be entirely appropriate, since the process is at least partially reversible *(vide infra).* Perhaps, the changes represent a combination of differentiation and modulation (for a review of these terms plus dedifferentiation, see Schjeide and Devellis[15]). Whatever their nature, the changes seen *in vivo* and *in vitro* are most probably examples of the same process. The relationship between this process and the activation or stimulation of mononuclear phagocytes remains to be clarified. Interaction of macrophages with various substances leads to enhanced functional states, characterized by ultrastructural changes similar to those reported here and variously termed *stimulation* or *activation* (for review, see Nelson[16]). These three alterations in macrophages might not necessarily by synonymous. For

example, the lysosomal hydrolases of cultured mcrophages have been segregated into at least three different classes, which differ significantly in their control mechanisms.[17,18]

The differentiation of mononuclear phagocytes occurring in the *M. tuberculosis* model was similar to that in the BCG model in some regards but differed significantly in one respect. In both models, differentiation was achieved by maturation of the mononuclear phagocytes and in both this proceeded through the same orderly sequence of changes. Indeed, the fine structure of differentiation in both was quite similar and, in turn, resembled that seen *in vitro* and *in vivo* in other circumstances.[14,19-22] However, as might be expected, the pace of differentiation in the two models was much different. While full differentiation was ultimately achieved in both models, it required 7 days in the *M. tuberculosis* model and 33 days in the BCG model (Table 1). The quickened tempo occurred not because the pattern or the extent of differentiation was altered, but rather because the entire process was accelerated. The transition time between each of the five stages was shortened, and the transition period from macrophage to immature epithelioid cell was particularly condensed. Despite varying circumstances, the differentiation of mononuclear phagocytes proceeds along relatively fixed lines and by definite stages. The tempo of the process, however, is subject to much variation. The factors controlling the rapidity of differentiation *in vivo* are not yet understood.

The fate of the granulomas and their constituent phagocytes is of in-

Table 1—Time of Appearance of Various Forms of Mononuclear Phagocytes in Two Types of Epithelioid Granulomatous Inflammation

Cell	Time of appearance in granulomas induced by BCG (days)	Time of appearance in granuclomas induced by *M. tuberculosis* (days)
Monocyte (promonocyte)	0	0
Immature macrophage (monocyte)	5	1
Macrophage	9	3
Immature epithelioid cell (stimulated macrophage)	21	5
Epithelioid cell (hypermature macrophage)	31	7

The data for BCG-induced granulomas is from Adams (Am J Pathol 76:17-48, 1974).[6]

terest. After their peak at 9 days, the lesions gradually waned and ultimately resolved. Epithelioid cells evolved into immature epithelioid cells and then into mature macrophages, while simultaneously the granulomas became smaller and less cellular. The mononuclear phagocytes then became immature macrophages, and the lesions were no longer granulomas but simple chronic inflammatory responses. Concomitant with these changes, the number of mycobacteria waned and ultimately became almost zero.

This sequence of events suggests that after mononuclear phagocytes *in vivo* have destroyed the ingested foreign substances, they can revert to less mature forms. This idea is supported by observations of Cohn and Benson *in vitro*.[23] In their studies, mononuclear phagocytes, already stimulated to differentiate *in vitro*, were removed from culture medium containing the stimulant. Within 24 hours, the number of lysosomes and their content of lysosomal enzymes had reverted to almost baseline values; reapplication of the stimulant led to restoration of differentiation. The present observations thus provide an *in vivo* counterpart of this phenomenon.

The fate of the mononuclear phagocytes in granulomas has heretofore been generally believed to be either death or division.[4,7] Dannenberg described the fate of mononuclear phagocytes in granulomas as death.[11] Ryan and Spector showed that short-lived macrophages in granulomas gave way to long-lived macrophages containing the irritant.[8,9] Spector and Lykke found that irritant-containing macrophages within granulomas die or divide to form small immature mononuclear phagocytes.[10] Papadimitriou and Spector showed that epithelioid cells have the same two fates.[12] All these studies thus suggest that macrophages, whether short-lived or long-lived, contain the irritant and persist until they die or divide. The present observations suggest a third fate for granulomatous macrophages—dedifferentiation (reversion to a less mature or less stimulated form). As the ingested irritant is degraded, highly mature mononuclear phagocytes change to a less mature state. The relative contribution of the three reactions in the total response to foreign irritants has not yet been established.

The ultrastructure of the response to *M. tuberculosis* has, surprisingly, been incompletely studied. Only the very early and very late responses are known.[24-28] The initial response has been established to be acute inflammation, as seen here.[24] The evolution of this initial response has not been traced, but the structure of mature tubercles is well known.[25-27] Epithelioid cells seen in the present study closely resembled those seen in other similar studies and those induced by other means (for review, see

Adams [6]). Two types of epithelioid cells, secretory and phagocytic, have been described by some observers.[26-27] The secretory type, principally seen in early and developing granulomas, resembles the immature epithelioid cells described here.[26-28] The phagocytic type, principally seen in mature granulomas, resembles the mature epithelioid cells described here.[26-28] Therefore, secretory epithelioid cells are probably epithelioid cells that are not fully developed or differentiated. Guzek has described variations in the number and shape of various organelles in epithelioid cells[26]; similar variations observed in the present study were judged to be within the expected range of variance. In summary: The present observations traced the sequential development of epithelioid granulomas induced by *M. tuberculosis*. In general, they agree with other studies of the late reaction, although they do not support the concept of two types of epithelioid cells.

The present study employed a previously described system to classify morphologically phagocytes as to degree of maturation.[6] The classification proved reproducible and readily applicable. It thus permitted quantitation of the maturity of various granulomas and, hence, direct comparison between them (Table 1). Other systems for classifying mononuclear phagocytes have been recently proposed.[14,29,30] In particular, an international study group distinguished between precursor cells, promonocytes, monocytes, and macrophages.[31] A rigid comparison between tissue phagocytes in inflammation and myeloid, hemic and tissue phagocytes in unstimulated hosts would not be profitable. It is kinetically and functionally apparent, however, that stem, young adult, and adult mononuclear phagocytes exist.[32,33] These correspond to the monocytes, immature macrophages, and macrophages described here, which might then be better termed promonocytes, monocytes, and macrophages. This implies immature mononuclear phagocytes are released from the marrow after inflammatory stimuli, a conclusion supported by kinetic analyses.[32,34] The present observations clearly demonstrate macrophages are capable of undergoing further maturation, so that stimulated or maturing macrophages (immature epithelioid cells) and hypermature macrophages (epithelioid cells) should be added to the above classification. The mononuclear phagocyte system might be classified as follows: precursor cell, promonocyte, monocyte, macrophage, stimulated macrophage, and hypermature macrophage (Table 1).

A conceptual model of granulomatous inflammation can be formulated on the basis of these studies. For reasons still poorly understood,[7] an acute inflammatory response evoked by foreign substances or by pathogens gives way to a chronic response—a loose collection of small mononuclear

cells which are predominantly mononuclear phagocytes.[32,33,35] If the substance persists and stimulates mononuclear differentiation, the young mononuclear phagocytes in the lesion (promonocytes and monocytes) mature into macrophages. The macrophages then become predominant, apositive, and aggregated into sheets or clumps (organized)—probably as a direct result of their differentiation. At this stage, a granuloma is deemed to be present. Upon further stimulation of differentiation, the mature macrophages become stimulated (immature epithelioid cells) and ultimately hypermature (epithelioid cells). Foreign body granulomas thus evolve into epithelioid granulomas, which persist until the evoking stimulus is destroyed or loses its powers to evoke differentiation. The hypermature macrophages then revert to mature macrophages and finally to monocyte-like forms; concomitantly, the lesions change from epithelioid cell granulomas to foreign body granulomas to chronic inflammatory responses. A *granuloma* is thus a tissue collection of partly or highly differentiated mononuclear phagocytes formed in response to a persistent injurious agent which can stimulate mononuclear differentiation. Nothing inherent in a granuloma's structure or type, therefore, specifies its etiology. The mononuclear phagocytes of foreign body and epithelioid granulomas differ only in degree and not in kind. Of course, the appearance of various granulomas depends not only on stimulatory properties of the evolving agent but also on other properties that produce necrosis, neutrophilic infiltration, etc., as well. Multiple agents, such as bacilli, lipids, and lymphokines, stimulate extensive differentiation *in vitro* and would be expected to evoke epithelioid granulomas *in vivo*.[2,5,35,37] Delayed hypersensitivity, for example, can easily be envisioned as evoking epithelioid granulomas but would not be the only stimulant for the formation of such lesions.[38] The differentiation of mononuclear phagocytes is central to the formation of granulomas.

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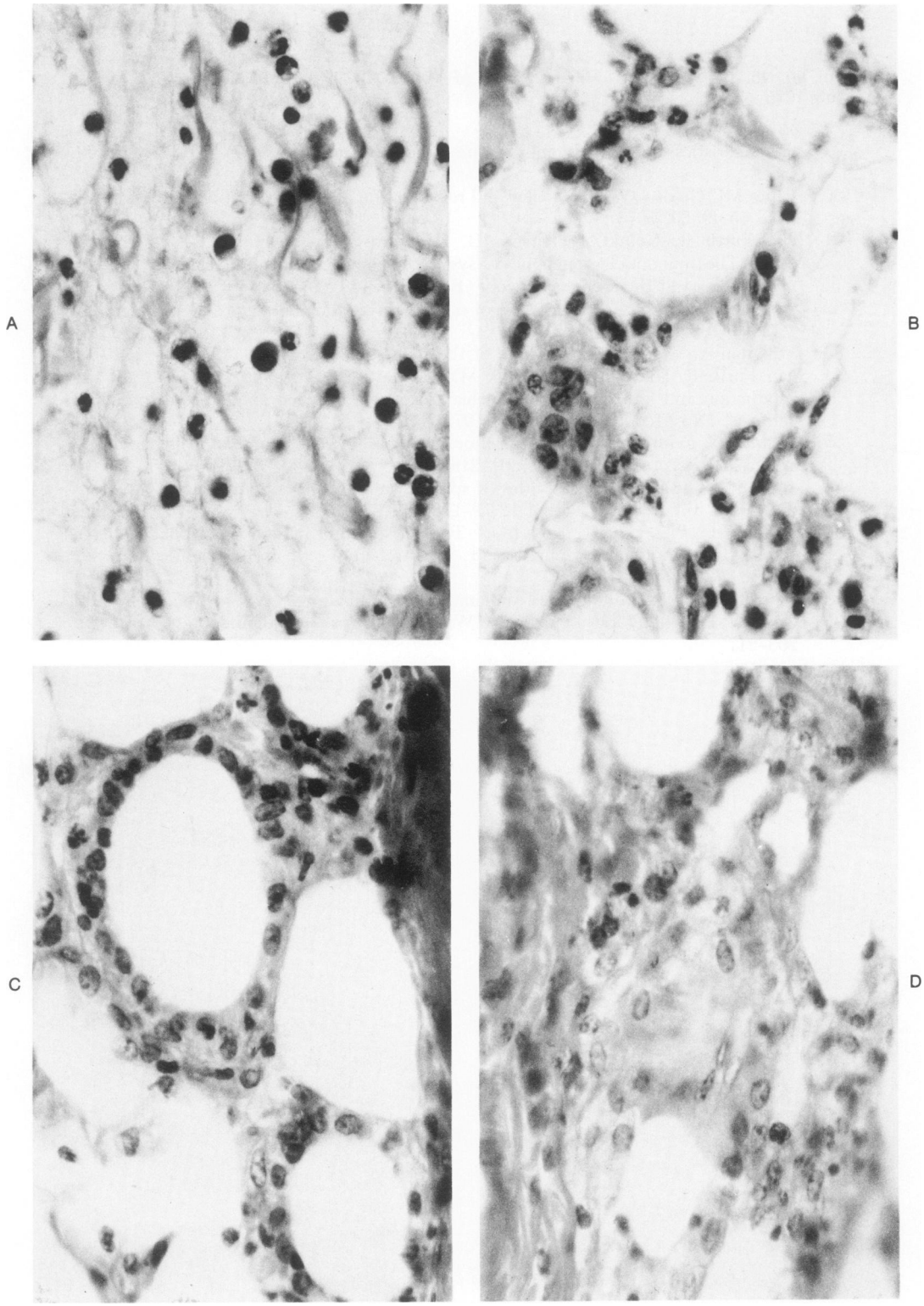

Figure 1—The initial stages of mononuclear differentiation *in vivo* induced by *M. tuberculosis.* **A**—Six hours. In an acute inflammatory response, scattered monocytes are seen. **B**—One day. Immature macrophages are scattered throughout the field; centrally, several more mature mononuclear phagocytes are clumped together. Note the increased cytoplasm and vesicular nuclei. **C**—Two days. The lesions consist mostly of mononuclear phagocytes in scattered clumps. **D**—Three days. Definite small granulomas, confluent sheets of mature macrophages, are present. Note the extensive amount of cytoplasm. (H&E, × 480)

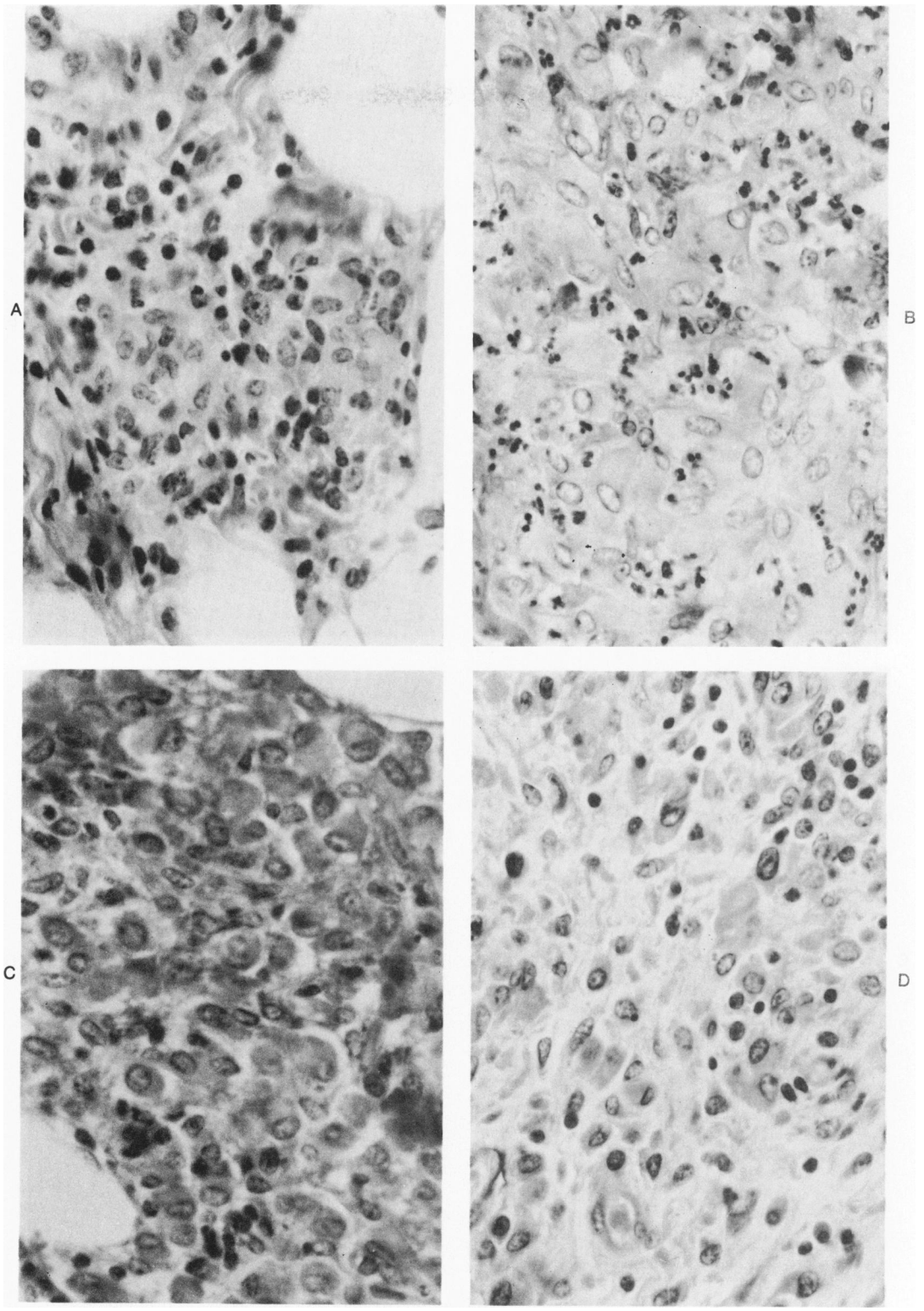

Figure 2—The full development and subsequent resolution of differentiation *in vivo* induced by *M tuberculosis*. **A**—Five days. A definite granuloma composed of immature epithelioid cells is seen. Note the blurred cytoplasmic borders. **B**—Seven days. A completely developed epithelioid granuloma is composed of coalescent sheets of mature epithelioid cells. The epithelioid cells have extensive cytoplasm, indistinct cytoplasmic borders, and large vesicular nuclei. **C**—Thirteen days. A loose collection of mononuclear phagocytes resembling a foreign body reaction is seen. Note the distinct cytoplasmic borders of the phagocytes. **D**—Fifteen days. A diffuse, light infiltrate of immature macrophages resembles banal chronic inflammation. (H&E, × 480)

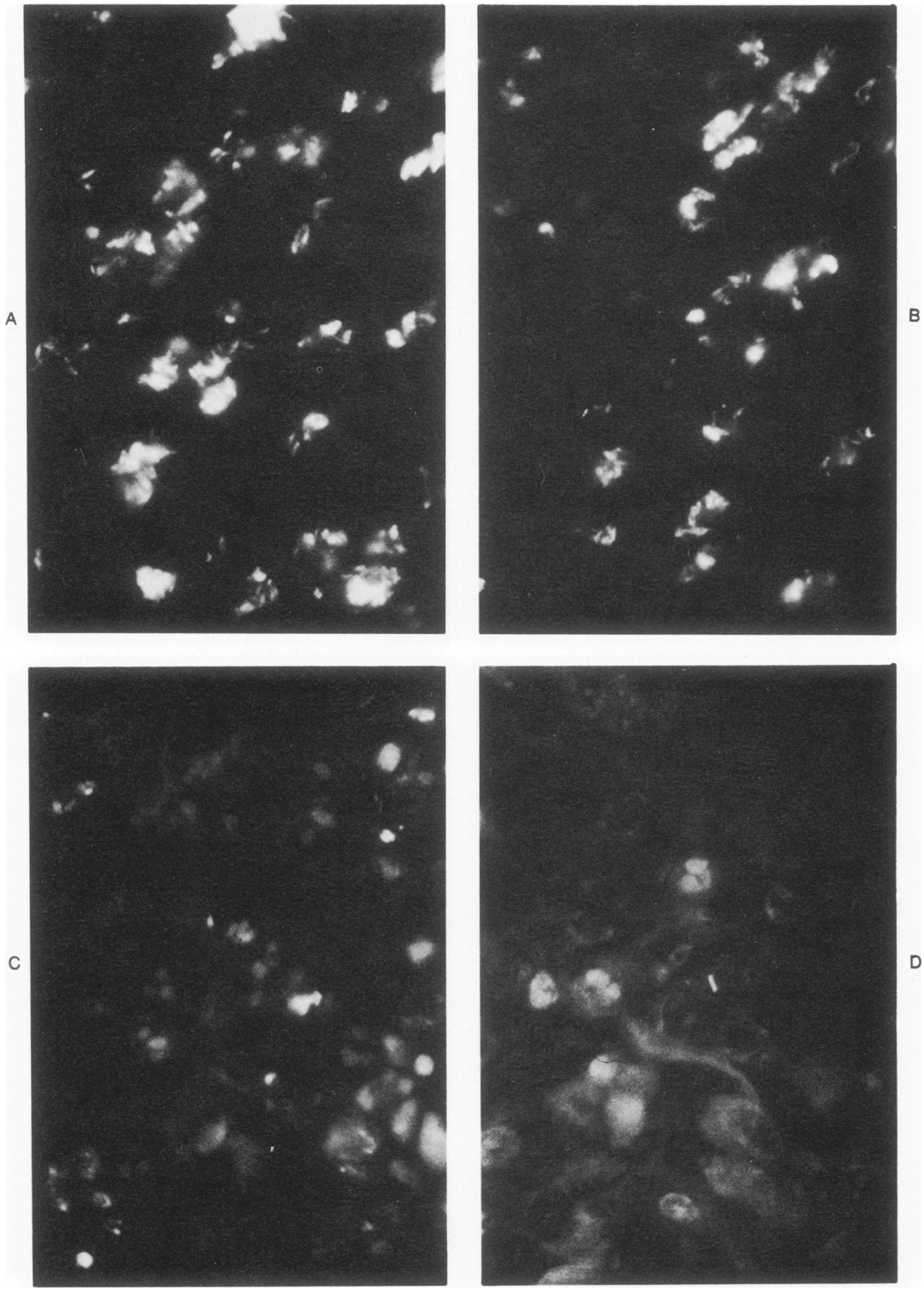

Figure 3—The number of mycobacteria present during developing and resolving granulomas induced by *M. Tuberculosis.* **A**—Three days. Large numbers of mycobacteria in dense clumps are present. All the organisms are located intracellularly. **B**—Nine days. A modest diminution in number of organisms is seen, and the bacilli are not as densely compacted. **C**—Thirteen days. The number of mycobacteria is dramatically altered: only a few scattered organisms can be seen. **D**—Nineteen days. One bacillus is seen at center; most fields at this time have none. (Fluorescent stain for mycobacteria, × 914)