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INVITED REVIEW

THE EXPERIMENTAL GRANULOMA. A HYPOTHESIS TO EXPLAIN THE PERSISTENCE OF THE LESION

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SUMMARY

Granulomatous inflammation is the morphological substrate of a variety of important infectious diseases such as tuberculosis, leprosy, schistosomiasis and others. Nevertheless, although many aspects of this special type of inflammation are known, fundamental questions concerning granuloma formation, persistence, fate and significance for host-parasite relationships still remain to be elucidated. In this brief review, the basic and more relevant literature related to experimental investigations on granuloma physiopathology is presented. Based on recent investigations performed in our laboratory showing that MDF (Macrophage Deactivating Fator) secreted by epithelioid cells and characterized as the calcium-binding protein protein MRP-14 deactivates activated macrophages, a hypothesis to explain the persistence of granulomatous inflammation is put forward.

KEYWORDS: Macrophage; Epithelioid cell; MDF; MRP-14; Granulona; Granulomatous inflammation.

INTRODUCTION

The last hundred years were not enough to draw a comprehensive picture of the mechanisms which govern the natural resistance to infection. Different areas of investigation have focused on the phenomenon and since then the inflammatory response, elicited by the presence of the parasite in a tissue, has been regarded as a major component of the process. With a few exceptions the first interaction between the parasite and the host takes place in an inflammatory *milieu*. The future course of infection depends largely on the effectiveness of phagocytic cells, specific antibodies, activated lymphoid cells and other unknown factors responsible for the killing and degradation of the parasite. Depending on the virulence of the parasite and the natural resistance of the host, the infection may be aborted, the host killed or, an unstable equilibrium between host and parasite be established with the appearence of signals and symptoms of the disease. If an unstable host-parasite equilibrium prevails, the inflammatory process originating in the infected organ or tissue may have peculiar characteristics which depend on the specific properties of the parasite and on the genetic background of the host. This is the case for microorganisms and certain materials capable of inducing a characteristic response of the affected tissue - the granulomatous reaction - described more than a century ago by Virchow. The granulomatous inflammation is the morphological substrate for many infectious diseases, such as tuberculosis, leprosy, brucellosis, schistosomiasis, sarcoidosis and a large number of visceral mycosis. In

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addition, noninfectious irritating agents such as talc, silica, berillum or zirconion are capable of evoking a granulomatous reaction in tissues to which they are applied. The large incidence of the granulomatous response in a variety of pathologic states is sufficient to encourage any effort to understand the unknown mechanisms which determine granuloma.formation. In this review the basic information which has been obtained from experimental studies on granulomatous inflammation will be summarized. Based on experiments showing that epithelioid cells secret a factor which deactivates activated macrophages, a hypothesis is proposed to explain the persistence of this peculiar type of inflammatory response.

Morphology of the granuloma

The typical granuloma induced by the *Mycobacterium tuberculosis* in man and laboratory animals, is a focal collection of mononuclear cells surrounded by connective tissue which includes fibroblasts, collagen fibers and newly formed vessels. A halo of small round cells, comprising newly arrived monocytes and a small number of lymphocytes is observed between the proliferating connective tissue cells and the focus of the lesion. Large mononuclear cells are arranged in a palisade formation which occupies the centre of the lesion. These cells exhibit a huge cytoplasm and a nucleus in which the chromatin is dispersed. They are referred to as epithelioid cells because of their resemblance to epithelial cells. Among the epithelioid cells, multinucleated cells or macrophage polykarya are observed and a central area of necrosis may occur [2,4]. The unique morphological characteristic of the granulomatous inflammation is the occurrence of epithelioid cells and the concentric appearence of the whole lesion. Some epithelioid granulomas are also heavily invaded by neutrophils [30] or by eosinophils [reviewed in ref. 12].

Granulomatogenic "Irritants"

Two basic categories of etiologic factors are recognized as inducers of granulomatous inflammatory responses. The first comprises inert substances that are unable to mount an immune response. These are artificially termed foreign-body types of agents and although non-immunogenic, their chemical properties render them able to generate granulomas which differ in evolution, dynamics, duration, severity and involution rate from infectious granulomas. There is a spectrum of responses to noninfectious "foreign bodies" ranging from a discrete or "quiescent" granuloma as induced by plastic beads [37], bentonite [37], carrageenan [71] in one pole, to a vigorous, "active" process as induced by silica [77], cord factor [7], streptococcal cell wall [78] on the opposite pole. The second category of granulomatogenic factors can induce immune reactions which drastically interfere with the dynamics and fate of the granulomatous lesion. To this category belong pathogens such as bacteria, fungi, viruses, protozoa and worms. Also metals such as zirconium and beryllium [38, 109] and artificially-made "irritants" composed of "foreign-body" particles, such as bentonite, coupled to an antigenic substance are included [37]. Despite their diversified nature, all granulomatogenic factors share one basic property, namely, they are poorly degradable materials [97, 98]. However, this property alone can not explain why a granulomatous lesion develops under the influence of such agents rather than a diffuse chronic inflammatory response.

Cells in Granulomatous Inflammation

Cells which comprise the typical tuberculous granuloma are macrophages, epithelioid cells, macrophage polykaria or inflammatory giant cells, and lymphocytes. (Fig. 1) The distribution of these cells within the lesion has been thoroughly described [2,4 reviewed in 1, 12]. However, some granulomatous lesions, as reported by ADAMS [12], do not contain all the cell types mentioned above. Furthermore, the lesions may exhibit infiltration of polymorphonuclear leucocytes as seen in fungal induced lesions [30, 72] or of plasma cells as is the case in schistosomotic lesions [12]. In our view, the epithelioid cell is the characteristic component of the granulomatous reaction [70]. Fibroblasts and newly formed vessels are seen to proliferate, particularly on the peripheral border of the developing tuberculous granuloma [25]. The extent to which this occurs, however, depends on both the nature of the causative agent and the animal species under study (BIRMAN & MARIANO, *unpublished results*).

The macrophage

The macrophage is the basic architectural and functional unit of chronic inflammation. While the morphology and function of resident, stimulated and activated macrophages from different body cavities has been extensively investigated in laboratory animals [reviewed in: 5,58,74,106,114], the characterization of macrophages in chronic lesions, particularly in

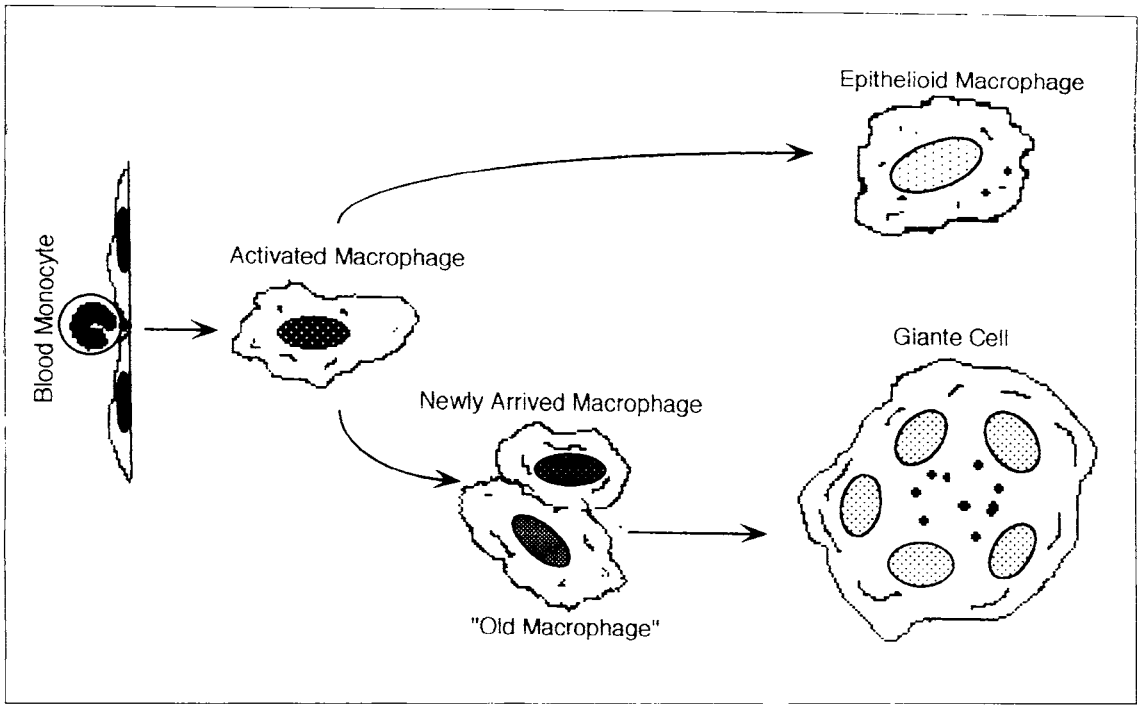

Fig. 1 - Schematic representation of macrophage transformation into epithelioid or giant cells in granulomatous lesions.

granulomas, has been hindered mainly because of the wide morphological variation found among these cells. ADAMS [2] made a carefull ultrastructural study of BCG-induced granulomas in guinea pigs and showed that newly arrived monocytes are initially simple cells which progressively increase their nuclear euchromatin content, develop prominent nucleoli, extensive cytoplasm, free ribosomes, abundant Golgi apparatus, mitochondria and large lysosomes, and finally acquire the typical morphology of inflammatory macrophages.

Origin of the macrophage - Cytokinetic studies have unquestionably established that macrophages participating in chronic inflammatory reactions originate from a precursor cell in the bone marrow, viz. the promonocyte which matures into the monocyte and reaches the injured tissue via the circulating blood [96, 112]. These findings allowed a new interpretation of the concept of "chronic proliferative lesion", according to which the inflammatory granuloma would be generated by the multiplication of tissue histiocytes [4]. The current view states that once at the inflammed site, macrophages may proliferate, the intensity of proliferation depending chiefly on the nature of the irritant [88]. Immunity may also play a part in the phenomenon through the enhancement of the rate of macrophage proliferation [8, 26]. Studies on BCG-induced granulomas in rats showed that macrophages divide only twice during their first week of residence in tissues and that local division does not substantialy contribute to the total number of cells in the lesion [88, 94].

Turnover - The development of a granulomatous lesion is a very active cellular process in which migrated monocytes may proliferate [88, 94], be actively recruited from blood [96, 112] or become long lived [88, 95], depending on the type of irritant used to induce the lesion. In terms of cell kinetics, granulomata represent a spectrum with turnover reaction at one end (tuberculous lesions and lesions induced by *B. pertussis* vaccine) and low turnover reaction at the other end (carrageenan-induced lesion) [95]. The turnover rate is reflected both in the speed of entry of new cells and the rate of division of existing cells [reviewed in 99]. Another relevant factor modulating cell turnover in a granulomatous lesion is the immune state of the host. An accelerated rate of macrophage turnover was observed in tuberculin hypersensitivity lesions induced in rabbits previously immunized with BCG [26]. Little is known, however, about the nature of the chemotactic factors which govern cell kinetic variations in the lesions.

Specific surface antigens - Mouse peritoneal macrophages exhibit specific antigens on their surface membranes. These antigens are detected by specific antisera and are reliable markers which allow distinguish macrophages from lymphocytes and other mononuclear cells [32, 45, 84]. However less clear results are obtained when antisera are tested against macrophages other than those obtained from the peritoneal cavity [66]. Macrophages at other sites may be more heterogeneous either because they are derived from different precursors or because they are at different stages of differentiation. Recent experimental evidence favors the later possibility (Discussed in Epithelioid cell - Differentiation antigens). Similar results were obtained with macrophages cultured "in vitro" [107]. The possible significance of these findings for the pathobiology of chronic lesions remains to be elucidated.

Surface receptors and phagocytosis - Macrophages are multifunctional cells. They are phagocytic and are capable of secreting a large variety of biologically active substances [reviewed in: 10]; they are involved in the processing and presentation of antigens to lymphoid cells; and in some way they regulate the immune response[32]. However, the ability to phagocytose and eventually digest foreign materials is its most characteristic activity. Phagocytosis by macrophages has been extensively studied "in vitro" [reviewed in: 102,103]. Cell membrane receptors involved in the phemomenon have been demonstrated on these cells. Whereas phagocytosis by macrophages has been thoroughly studied "in vitro", little is known about the phagocytic activity of macrophages in chronic inflammatory lesions. Subcutaneous implantation of glass coverslips in the mouse induces a foreign-body type of inflammatory reaction which has proved useful for the study of the phagocytic activity of macrophages "in vivo". Adherent cells stick to the glass surface and can be collected for microscopic observation [88, 112]. With this technique phagocytosis mediated by Fc, C3b, or other surface receptors, could be demonstrate with cells that were recovered from coverslips implanted in mice for less than 14 days. Later, most macrophages appear to loose surface receptors with the consequent impairment of phagocytic activity [64, 80]. Similar findings were observed in chickens [46]. Results obtained in guinea-pigs indicate that if *M. tuberculosis* is subcutaneously inoculated with the coverslip, macrophages on the glass surface are significantly more phagocytic, provided the animal was previously immunized with CFA. This increased phagocytic activity is maximal at day 3 to 6 and then declines to control levels on the fourteenth day post infection [65]. The increased phagocytic activity of macrophages in delayed hypersensitivity mediated lesions was also observed in cyclophosphamide-treated mice immunized with sheep red blood cells [9].

Epithelioid cell

Inflammatory macrophages may develop into epithelioid cells which are the characteristic components of the granulomatous inflammation. The epithelioid cell is recognized under the light microscope as possessing a pale, elongated nucleus and a cloudy eosinophilic cytoplasm whose outline can merge with that of the neighboring cells. Epithelioid cells arrange themselves either in layers or form discrete aggregates. They are found mainly in the central part of a lesion or in surrounding necrotic areas [4, reviewed in 39]. Observed under the electron microscope, epithelioid cells resemble large polygonal macrophages with a large euchromatic nucleus, prominent nucleoli, and an abundant cytoplasm filled with many lysosomes and mitochondria. An extensive endoplasmic reticulum is seen, and bundles of microfilaments may be observed. The cells appear closely associated, and are interlocked by twinning pseudopodes [2, 13]. A characteristic type of cytoplasmic granules has been described in epithelioid cells [111].

Origin and differentiation - It is now clear that the epithelioid cell do derive from the macrophage. PAPADIMITRIOU & SPECTOR [79] implanted pieces of cellophane into the subcutaneous tissue of mice and recovered cells morphologically alike to the epithelioid component of granulomas. Epithelioid-like cells were also described in foreign-body lesions induced in mice by subcutaneous implantation of glass coverslips [64, 111]. In a detailed morphological study of BCG-induced granulomas in guinea pigs, ADAMS [2] reported the changes undergone by monocytes during their transformation into epithelioid cells. Five distinct stages were then sequentially defined in the course of cell differentiation: monocyte, immature macrophage, macrophage, immature epithelioid cell, and epithelioid cell. Immature epithelioid cells are observed in 21 day old lesions. Epithelioid cells are first recognizable after approximately 30 days. These observations were confirmed in experiments in which lesions were induced by killed tubercle bacilli on CFA-sensitized guinea pigs [13].

Epithelioid cells appear to have a reduced capacity to proliferate as compared to newly arrived macrophages [108].

Differentiation antigens - SORG and his group [76] cloned two calcium-binding proteins from the supernatant of MIF reaction. These proteins which belongs to the family of the S-100 calcium-bindind proteins [56], named MRP-8 and MRP-14 (MIF Related Proteins with molecular weight of 8 and 14), were shown to be present in neutrophils and monocytes [115]. Macrophages and neutrophils express these proteins in different types of inflammatory reaction. Nevertheless, as macrophages mature in the inflammatory foci they no longer express these markers [86]. The real role played by these proteins in leukocyte physiology and pathology remains to be determined [reviewed in 49]. Nevertheless, DELABIE et al. [31] demonstrated that, contrary to observed in inflammatory macrophages in which the simultaneous expression of MRP-8/14 is a constant, only MRP-14 can be detected in the cytoplasm of epithelioid cells of typical tuberculous granulomas in man. According to these authors, "foreign-body" granulomas do not present this pattern of reaction. Using monospecific antibodies directed to these proteins in immunostained preparations, we have shown that epithelioid macrophages from coverslips implanted into the subcutaneous tissue of mice, a typical foreign-body granuloma, also express MRP-14 but not MRP-8 (MARIANO et al. *in preparation*). Yet, we have confirmed the preponderant expression of MRP-14 in epithelioid cells from different types of granulomas in man including "foreign-body" granulomas induced by the presence of silk suture thred in the tissues. These finds undoubtedly opens the opportunity to revise the concept of "epithelioid cell" in chronic inflammation.

Phagocytosis - The ultrastructural organization of epithelioid cells suggests that they are poorly phagocytic. Indeed, PAPADEMITRIOU & SPECTOR [79] found that cells developing on implanted cellophane sheets and endowed with morphological characteristics of epithelioid cells were less phagocytic than their macrophage precursors. On the other hand, they exhibit active pinocytosis and exocytosis of colloidal gold particles. Inflammatory macrophages, which adhere to the surface of subcutaneously inserted coverslips, express Fc, C3b and other surface receptors and are actively phagocytic via these binding sites [64]. However, about 50% of the mono and multinucleated cells which are recovered from 21 day old coverslips no longer express these surface receptors and no longer phagocytose test particles. Ultrastructurally, these cells have the classical morphological characteristics of epithelioid cells. Impaired phagocytic activity has also been observed in giant cells. This appears to suggest that the macrophage-derived giant cells may also undergo an epithelioid transformation. One of the unsolved problems regarding the biology of epithelioid cells is whether this type of macrophage transformation is a permanent and stable phenomenon. Experimental evidence shows that the impaired phagocytic capacity of epithelioid cells may be reversed. When epithelioid macrophages and macrophage polykarya are incubated with a mixture of antibody coated sheep red blood cells and levamisole they actively phagocyte the complex, indicating that at least in some circumstances epithelioid cells may regain the ability to phagocytose [60].

Epithelioid cell function - The function(s) of epithelioid cells in granuloma physiopathology is still obscure. Based on the morphology of these cells PAPADIMITRIOU & SPECTOR [79] have suggested that secretion of biologically active factors is the main function of these cells. CAMARERO et al [17, 18] demonstrated that "pre-epithelioid" and epithelioid cell populations on the surface of implanted glass coverslips in mice, secret a factor(s) which blocks O_2^- release from newly arrived macrophages. Besides this, secretory capacity of epithelioid cells were shown to control the rate of multiplication of *Mycobacterium tuberculosis*-BCG in granulomas induced by this bacteria in the hamster check-pouch [91].

"In vitro" culture of epithelioid cells - When mammalian macrophages are cultured "in vitro", the typical epithelioid transformation is not observed. Chicken blood mononuclear cells were cultured "in vitro" and cells with typical epithelioid morphology were found in the culture [105]. Subsequently, however, it was observed that more than 90% of the mononuclear cells that adhere to culture dishes were thrombocytes and not monocytes as previously considered. Chicken thrombocytes can exhibit a behaviour analogous to mouse inflammatory macrophages because they are also able to fuse and form giant cells which then loose surface receptors involved in phagocytosis [46]. Attempts have been made to maintain an "in vitro" culture of cells adherent to coverslip-implants in subcutaneous tissues of mice. The attempts were unsuccessful: after 3 days in culture, adherent macrophages and macrophage polykarya begin to round up, detach from the

glass surface, and start to show morphological signs of cell degeneration (personal observations). Further investigations are needed to understand this phenomenon.

Giant cell (Macrophage polykarion)

The presence of multinucleated cells is a constant feature in certain types of chronic inflammatory lesions. These cells are seen in tuberculous lesions in man and bovine cattle. At least two cell categories can be distinguished by their morphological characteristics: the Langhans giant cells which have nuclei disposed peripherally so as to form a crown-like figure, and the foreign-body giant cells with nuclei dispersed throughout the cytoplasm in a somewhat less organized manner. The Langhans cell type probably derives from the foreign-body type cell and represents a more advanced/mature cell stage [61]. The significance of cell multinucleation in these lesions is still obscure. In tuberculous lesions, its occurrence appears to be species-dependent since multinucleated cells are rarely observed in dogs and cats [36]. In experimentally induced tuberculous lesions, multinucleated cells are seen in guinea pigs and in the rabbits, but are less frequent in mice, rats and hamsters (BIRMAN & MARIANO, *unpublished results*).

Origin, differentiation and fate - The macrophage origin of multinucleated cells in chronic inflammatory lesions is now well established. When bone-marrow macrophage precursors are labelled with tritiated thymidine (3HT) and a chronic lesion is then induced, the isotopic marker can be detected in the nuclei of giant cells which appeared during the development of the granuloma [44, 61, 88]. Macrophage fusion is thought to be the basis for the origin of multinucleated cells. Cytoplasmic contact and fusion between macrophages is observed in electronmicrographs of the lesions. Since fusion processes appear to be a rapid event, they are not easily observed under the microscope. Giant cells may also be formed through repeated nuclear division without cytokinesis. However, labelling experiments have shown that synchronous and asynchronous DNA synthesis occurs in only a small number of multinucleated cells. These nuclei may enter the mitotic phase of division, with their chromosomes presenting typical structural aberrations. At the end of the mitotic phase abnormal poliploid nuclei result [61]. Once multinucleated cells are formed, newly arrived macrophages may be incorporated into the syncytium, thereby increasing the total number of actual nuclei. Since pretreatment of experimental animals with colchicine induces the transformation of Langhans cells into foreign-body cell types, probably by disorganizing microtubular structures in the cytoplasm, it was suggested that foreign-body giant cells really mature into the Langhans type. This proposed sequence of events is further supported by the fact that foreign-body giant cells are the first to appear on the surface of subcutaneously implanted coverslips while the typical Langhans cells only appear at a later stage. Ultrastructural observations are also consistent with this interpretation [61] despite some conflicting results [111]. Although two basic types of giant cells have been distinguished, the giant cell population as a whole exhibits considerable morphological variation. It has been shown that when glass coverslips are left in the subcutaneous tissue of mice for more than 21 days, peculiar Langhans giant cells appear on the glass surface. When observed by phase contrast microscopy they look globous and supported by a cytoplasmic holdfast, resembling a "crystal ball". These cells share some ultrastructural and functional characteristics with epithelioid macrophages [64]. The fate of multinucleated cells in chronic inflammation has not been elucidated to date, although it is known that when they are inserted into the subcutaneous tissue of mice inside millipore diffusion chambers they have a life-span of about 6 days [61].

Giant cell formation - One of the models used for studying giant cells in chronic inflammation is that of coverslip-induced lesions. Using mice and rats as experimental animals it was found that multinucleated cells appear on the glass surface four days after coverslip implantation. The determining factor(s) for cell fusion is not known. At last in this model in which activated lymphocytes do not participate in the turnover and differentiation of the lesion, IL-4 [69] and other lymphokines can be ruled out as factors which induce macrophage fusion. One hypothesis is that giant cells might result from fusions of senescent macrophages. To test this hypothesis, 48-hour coverslips were put inside millipore chambers, and these then inserted back into "donors" from which the coverslips had been recovered. Removal of the chamber at different time intervals showed that giant cell formation did not occur. However, when holes were made in the chambers to allow newly arrived macrophages to enter the chamber, then macrophage polykarya were formed. This finding indicates that fusion might have occurred between both, the "old" and the "young" populations of

macrophages [61]. The "old" population might consist of epithelioid cells. Nevertheless, subsequent data showed that epithelioid cells only appear on the glass surface after 14 to 21 days of inflammation [64], whereas multinucleation is first observed on the fourth day after coverslip implantation. Because macrophages recovered from 48 hour coverslips have chromosomes with typical aberrations it was suggested that cell fusion occurs when newly arrived macrophages recognize these changed cells [61]. CHAMBERS [19,20], however, challenged this hypothesis and presented evidence that giant cell formation results from the attempt of two or more macrophages to phagocytose the same particle, with the consequent fusion of their plasma membranes. Another intriguing problem is why giant cells are formed in some chronic lesions. It was proposed that the turnover of the lesion might be the real factor [61,99]. Giant cell formation was almost abolished when BCG was simultaneously inoculated into the subcutaneous pocket where glass coverslips were inserted [9]. Taking this experiment further, the same authors compared the effects of inoculating BCG, heat-killed BCG and carrageenan; the previous observation that alive BCG blocks giant cell formation was confirmed, heat-killed BCG was found not to interfere with the process, and carrageen greatly increased both the number and size of multinucleated cells, a set of results which indirectly suggests that the cell turnover of a granuloma may indeed be a determining factor for the formation of macrophage polykarya [8].

Surface receptors, surface antigens and phagocytosis - In granulomas induced by mycobacteria, fungi or even inert particles, the causative agents are frequently seen within the cytoplasm of multinucleated cells. In the past, the capacity of these cells to phagocytose was a matter of debate, since the material found within their cytoplasm could have been ingested by parent macrophages before cell fusion had occurred. In recent years, experimental evidence has been accumulated which supports the view that macrophage polykarya are one of the cellular components of the phagocytic system; giant cells have been shown to phagocytose heat-killed mycobacteria both "in vivo" and "in vitro" [61] and they also express Fc and C3b surface receptors found in macrophages. PAPADIMITRIU et al., [80] showed that the phagocytic ability of giant cells was inversely related to the size of the polykaryon i.e., as the number of nuclei increased, the capacity to phagocytose decreased. These observations suggest that the incorporation of macrophages during giant cell formation is accompanied by a progressive loss of surface receptors involved in phagocytosis. Indeed, it was found that, the great majority of the giant cell population that was recovered from coverslips subcutaneously implanted in mice for more than 21 days, did not express surface receptors for the Fc region of heterologous IgG or for the C3b component of the complement system. The loss of these receptors, together with evidence obtained from ultrastructural studies, led to the suggestion that macrophage polykarya are transformed into "epithelioid polykarya", a phenomenon analogous to the epithelioid transformation of mononuclear phagocytes [64]. However, "in vitro" it was found that when levamisole is added to the culture medium, the "paralyzed" multinucleated cells do reexpress Fc receptors [60]. Although the above data show that inflammatory macrophage polykarya are phagocytic cells, the primordial function of these cells remains to be elucidated.

Lymphocytes

It is now unquestionable that lymphocytes play a central role in a large variety of inflammatory conditions by mediating local or systemic immune responses. There is also evidence to show that these cells may modulate non-immune inflammatory lesions [42]. Ultrastructural observations, and studies using immunocytochemical methods, have shown that lymphocytes and plasma cells are found in different types of granulomas [110]. The presence of Migration Inhibition Factor (MIF) and eosinophil chemotactic lymphokines in liver granulomas isolated from schistosome-infected mice indirectly suggests the existence of T-cells in these lesions [11]. Using immunofluorescent methods it was shown that the T and B lymphocytes comprise less than 0.1% of cells in coverslip-induced granulomas in mice. Similar results were observed when coverslips and PPD were inserted into animals previously sensitized with CFA (MARIANO & RIBEIRO DOS SANTOS, *unpublished results*). These observations reinforce the concept that macrophages are the prevalent mononuclear cell population which migrate into chronic lesions, and that a small number of sensitized lymphocytes is enough to change the turnover and functional activities of macrophages in inflammed tissues [67].

Granulocytes

Neutrophils come into play during the early stages of a forming tubercle or tuberculous granuloma [4]. Large

numbers of these cells are also present in early lesions evoked by BCG in different animals species (BIRMAN & MARIANO, *unpublished results*). With the development of the granuloma, neutrophils tend to disappear. Despite the quantitative reduction, neutrophils play a significant role in liquefying tuberculous granulomas, leading to the formation of cavities and dissemination of tuberculous bacilli [41]. These cells are also present in *P. brasiliensis* granulomas. The role of neutrophils in granuloma formation has not been fully established. Some results show that their migration to the site of inflammation by chemotactic attraction is not dependent on the complement system [15] but on factor(s) released by macrophages [14]. Eosinophils are moderately phagocytic cells [53] which may have an important influence on the modulation of histamine release by mast cells and basophils [51]. These cells have been traditionally linked with allergic reactions and parasitic infection. Eosinophilia and accumulation of lesion-associated eosinophils in hepatic schistosoma granulomas seem to be mediated by T-cell derived lymphokines [50]. Eosinophils are present in different types of granulomatous inflammation, and are a constant feature of the early stages of experimental hepatic granulomas in schistosoma-infected mice [92]. The migration of these cells to such lesions is a T cell-dependent phenomenon, but their role is still obscure. The idea that eosinophils might have some parasiticidal action comes from "in vitro" experiments, which show that they exhibit an antibody-dependent cytotoxic effect on schistosome eggs [52]. Despite the occurrence of large numbers of basophils in Crohn's disease granulomas, little is known about the relevance of these cells in other types of hypersensitivity granulomatous lesions [12].

Vascularization of granulomas

The limits of a granulomatous lesion are not always sharply defined. Granulomas are surrounded by a capsule of proliferating connective tissue composed of fibroblasts, collagen fibers, amorphous substances and newly formed vessels. Connective tissue proliferation and the formation of new vessels are important events in granuloma formation. The connective tissue is the source of cells recruited to the lesion, and the newly formed vessels provide oxygen and nutrient supply to cells present in the granuloma. Spontaneous tuberculous granulomata in man are avascular, i.e. no vascularization occurs within the mass of macrophages and epithelioid cells, which make up the central part of the lesion [4]. This observation suggests that either cells within the granuloma (epithelioid cells) inhibit neovascularization or stimulatory substances to endothelial cell multiplication are not secreted, for instance, by macrophages. [104]. However, little is known about vascularization of other types of granulomas. COURTADE et al., [25] studied the vascularization of BCG induced granulomas in rabbits. They showed a 60% increase in capillary density in non necrotic lesions. This increase was constant in necrotic lesions and in lesions induced by tuberculin. Little information is available concerning the possible influence of the connective tissue on granuloma formation. MELRO & MARIANO [70] described a typical granulomatous response around *Schistosoma mansoni* eggs, which were "freely floating" in the peritoneal cavity of experimentally infected mice. These observations suggest that the connective tissue does not play a fundamental role in granuloma formation and organization although an extracellular matrix, not yet fully characterized, was observed in these "free floating" lesions. A similar phenomenon as that described by MELRO and MARIANO [70] is observed in lower animals. When crustaceans, for instance, are invaded by living or inert foreign bodies, the hemocytes react against them by means of phagocytosis and multicellular reaction resulting in "encapsulation" or "nodule formation" [85]. These lesions are structurally similar to granulomas observed in mammals suggesting that this type of cellular response to foreign bodies is a very old phenomenon in phylogenesis.

Granuloma formation

Foreign-body versus Immune Granuloma

Little is known about the mechanisms which determine macrophages to organize into a focal and compact lesion to form a granuloma and not to diffuse in chronic inflammatory process. One possible explanation is that granulomatogenic agents behave as chemoattractants or induce the generation of chemotatic factors from tissues, thereby centripedally directing the cells to surround and to envelop the irritant. If the causative agent is particulate and larger than the phagocytes as is the case for bentonite, plastic beads or schistosome eggs this behaviour of the cells seems obvious. However, this does not appear to be the case in other circumstances. It has been long known that when virulent tuberculous bacilli are inoculated into the subcutaneous tissue of a non-sensitized guinea pig, the bacteria is detected in regional lymph nodes within a short interval of time [16]. At the point of inocu-

lation a mild acute inflammatory reaction will develop followed by migration of polymorphonuclear leukocytes and monocytes which phagocytose the inoculated bacteria. The neutrophilic migration subsides and macrophages which phagocytosed the bacilli start to aggregate, resulting in a focal lesion. In about one week a typical granulomatous lesion develops. The mycobacteria are then limited to the necrotic area of the granuloma and are present in small numbers within epithelioid cells. They are not detected at the halo of newly migrated cells which delimitates the edge of the lesion. Inert particles (silica, glass, talc, cotton) if injected into the connective tissue of experimental animals will also evoke a transient acute inflammatory response that is followed by an intense macrophage accumulation and granuloma formation. Macrophages, epithelioid cells and giant cells are found in these foreign-body granulomas [reviewed in 12]. In a series of elegant experiments WARREN [113] showed the influence of the immune system, on the evolution of schistosome egg granulomas in mice. Based on these data he classified granulomas in immunological and non-immunological granulomas. This classification suggests that the formation of the immunological granulomata is entirely dependent upon sensitized and activated T cells. In support of this hypothesis BOROS & WARREN [11] showed that the injection of bentonite particles covered with different antigens into the circulation of sensitized animals induces, in the lungs, a mononuclear cell collection around the particles only when the animals were previously sensitized to the same antigen. More recently it has been shown that "granuloma" develops "in vitro" when sensitized T cells are put in mixed cultures of macrophages and schistosome eggs [6, 33, 34, 57]. These experiments suggest that antigen activated T lymphocytes secret lymphokines which attract macrophages and hold these cells together resulting in the granuloma formation. This mechanism of "immune-granuloma" formation can be questioned. In the experiments of BOROS & WARREN [11] and in those of DOUGHTY & PHILLIPS [33,34] the morphology of the lung lesions or of the cell collection around the eggs "in vitro" can not be classified as granulomatous lesions. The presence of epithelioid cells in these experimental situations has not been demonstrated, and a simple focal mononuclear cell collection should not be regarded as a granuloma. The same argument applies for focal hepatic lesions induced in mice by *L. donovani* [68]. Even considering the limitations of these models a series of experiments demonstrate that interleukin-1 but not interleukin-2 plays a role in the initiation and development of pulmonary granulomas in mice [54]. Evidences that interleukin-1 and TNF-α are envolved with the phenomenon of macrophage aggregation around inert particles in vitro has been recently demonstrated [90]. The participation of TNF-α in the genesis of BCG-induced granuloma in mice has been clearly demonstrated by KINDLER et al. [55].

Another set of experiments also rules out the participation of T-cells in granuloma formation. Nude mice develop granulomatous lesions when injected with BCG [3] and when inoculated with schistosome these mice are able to mount a granulomatous response around the eggs [40]. CHEHL et al. [21] have also shown that the inoculation of *M. leprae* in nude mice induces typical granulomatous responses in these animals. Interestingly, about two months after inoculation of the bacteria, the lesions are no more granulomatous but resemble those observed in lepromatous patients. Another evidence that granuloma formation does not depend on activated T cells comes from the work of MELRO & MARIANO [70] who showed a typical granulomatous arrangement of mononuclear cells around viable schistosome eggs inoculated into the peritoneal cavity of noninfected mice. The "free floating" granulomas were observed 48 hrs after the inoculation of the eggs. Finally, the inoculation of BCG in the cheek pouch of hamsters which lacks lymphatic drainage, induces typical granulomas despite the fact that the animals do not react to the intradermal injection of PPD [91]. More recently, NORTH et al. [75] demonstrated that typical granulomatous response is observed in the liver of SCID mice inoculated with BCG. These experiments clearly show that neither T nor B lymphocytes play any role in the genesis of granulomas. These observations, added to the fact that a foreign body, such as a glass coverslip can induce epithelioid transformation of macrophages [64] and giant cell formation [22, 87], the two basic cellular components of the granulomatous response, strongly suggest that granuloma formation is a macrophage phenomenon influenced by but not dependent on lymphocytes. However, the chemical characteristics of the granulomatogenic "irritant" which induces macrophage aggregation and transformation into epithelioid cells, remains to be elucidated.

Immune modulation of the granuloma

While granuloma formation largely depends on

the nature of the "irritant" which determines macrophage aggregation and modulation into giant cells and epithelioid cells, there is no doubt that the immune responses do modulate the expansion and the morphology of lesions. The pioneer work of WARREN [113] on the influence of the immune system on the schistosome egg granuloma indicate that the volume of lung granulomas, induced by intravenous injections of schistosome eggs, is increased when sensitized lymphoid cells are transferred to non sensitized recipients. Later, BOROS and colleagues [reviewed in 12] reported that transfer of lymphoid cells from animals in the late stages of infection, reduces the volume of schistosome egg granulomas. The phenomenon has been analyzed in more detail to show that the inflammatory response to schistosome eggs involves a dynamic interaction of lymphokine-secreting Lyt 1+ effector and Lyt 2+ suppressor T cells. Some evidences suggest that the suppressor T cells are recruited in a feedback manner by Lyt 1-, Qa+ helper cells and exerts their effects through soluble suppressor factors which can regulate lymphokine production [22,24]. PERAÇOLI et al. [81] observed a clear correlation between the humoral or cellular response to *Paracoccidioides brasiliensis* in hamsters and granuloma morphology. When cell mediated immunity, as evaluated by lymphoid blast transformation "in vitro" is high, a typical granulomatous response to the fungi is observed, i.e., the granulomas are dense and well organized. Conversely, when cell mediated immunity decreases, with the concomitant increase of specific antibody titres, the granulomas are loose and not well defined. This model closely ressembles the morphological and immunological characteristics of leprosy in man as far as cell mediated immunity, humoral response and granuloma formation are concerned [35]. de BRITO et al. [27,28,29] have brought evidences that antibody-mediated immune response might participate in the pathogenesis of schistosomal granulomas by permeating the mantel of epithelioid cells and neutralizing small doses of egg antigens shaded by living eggs. For these authors, this might prevent large amounts of immune complexes formation and consequent increase in tissue destruction.

The special case of leprosy granuloma

Since the discovery of the etiologic agent of lepra in man by HANSEN [48] many attempts to reproduce the disease in experimental animals have failed. Some progress has been achieved after the work of SHEPARD [89] who succeeded in growing the bacilli in the foot pad of Balb/c mice. In addition *M. leprae* was found to grow and disseminates in athymic nude mice much more vigorously than it does in the heterozygote counterpart [21]. In both experimental models, the bacilli induce a granulomatous response which, however, does not keep a close morphological correlation to those observed in tuberculoid leprosy in man. The typical peripheral nerve damage observed in tuberculoid leprosy in man is not observed in these models. In contrast to what is observed after the inoculation of other mycobacteria in the subcutaneous tissue of experimental animals, the injection of dead *M. leprae* does not induce a significant inflammatory response. When live bacilli are inoculated even in Balb/c or Nu/Nu mice, about two months have to elapse before the bacilli start multiplication, followed by an intense inflammatory response. Although the mechanisms involved in the initiation of the inflammatory response to live *M. leprae* are not known, it appears to be a T-independent phenomenon since it does occur in athymic mice [21]. Experiments from this laboratory demonstrate that dead *M. leprae* and particularly its lipidic components blocks phagocytosis by macrophages "in vivo" suggesting that the bacteria induces immune-repulsion in the host [73]. We have also shown that the inoculation of dead *M. leprae* from human origin (Lepromin) in the foot pad of hamsters previously immunized with BCG, induces, after 16 days of inoculation, a typical granulomatous response which surrounds myelinated peripheral nerves [59]. Although nerve damage could not yet be demonstrated, this model seems useful for the study of the basic mechanisms by which granuloma formation is induced by *M. leprae* antigens.

Concomitant immunity and a hypothesis for granuloma persistence

The classical Koch's phenomenon is a biological paradox not yet solved. If a guinea pig is inoculated with live *M. tuberculosis* the animals will die in three to four weeks. Nevertheless, if an inoculated animal is subcutaneously reinoculated with the same microorganism, a flourishing inflammatory response will appear at the site of inoculation which may become necrotic in a few days and heals in about two weeks. No bacteria are found in the regional lymph nodes. Therefore, the animal is competent to kill the microorganism after the second but not after the first inoculation. The basic difference between both circumstances is that following the first inoculum multiple granulomas develop whereas the second inoculation induces a chronic

inflammatory reaction with no granuloma formation. This phenomenon, named concomitant immunity, occurs not only in infectious diseases [82] but also in some types of transplantable tumors [43]. While the second inoculation could be interpreted a classical T cell mediated delayed type of hypersensitivity, this latter phenomenon is not always effective in eliminating the bacilli within the granuloma. Helper and suppressor T cells have been shown to populate the mantel surrounding infectious granulomas but not the centre of the lesion and it has been suggested that these cells might modulate macrophage functions and turnover [11, 100, 101]. The reason why newly arrived macrophages and lymphocytes do not penetrate into the mass of epithelioid cells which have taken up the bacilli in their cytoplasm is not known. A plausible explanation for the failure of immune effector mechanisms to eliminate the bacilli within a granuloma is that the cells inside the lesion might deactivate lymphoid cells and/or newly arrived macrophages. Evidence for this hypothesis has recently emerged [17, 18]. Macrophages on the surface of glass coverslips subcutaneously implanted in mice for 7 days, spontaneously release superoxide anion in appreciable amounts. If the coverslips are removed after 14 and 21 days of implantation, the cells on their surface no longer release detectable amounts of this radical, even following stimulation with phorbol mirystate acetate. When a delayed type of hypersensitivity reaction was induced around the 14 days old coverslips, to recruit macrophages, the histological analysis of the coverslips showed the presence of newly arrived cells but no O_2^- release has been detected. When 5 days old lesions were tested, the induction of a delayed type of hypersensitivity reaction, around the coverlips, induced a marked increase in superoxide liberation by the cells. These results suggest that the "old" cells in the lesion might be responsible for deactivating the newly arrived cells. To analyze this hypothesis, 5 days old coverslips were co-cultured with 14 day old coverslips and the release of O_2^- was measured. Under these circumstances, superoxide anion release by the cells on coverslips removed after 5 days was completely inhibited suggesting that the "old" cells secrete a factor or factors which deactivate inflammatory macrophages despite the activation of these cells by lymphokines secreted by activated T lymphocytes. This factor was named "Macrophage Deactivating Factor, MDF". MDF was partially characterized as a low molecular weight protein (12 kDa), termo resistant and sensitive to tripsin and pronase [17]. We have recently produced a policlonal antiserum directed to antigens secreted by murine epithelioid macrophages [63]. These antibodies immunostained epithelioid cells from coverslips implanted for more than 14 days in the subcutaneous tissue of mice as well as epithelioid cells from BCG-induced granulomas in the same species. They also blocked the MDF deactivating activity "in vitro" and decreased the volume of BCG-induced granulomas in the foot pad of mice when injected intraperitonealy in these animals. Following the observations of DELABIE et al. [31] who showed that epithelioid cells from tuberculous but not from foreign-body granulomata in man selectively express MRP-14 but not MRP-8 calcium-binding proteins [76] in their cytoplasm, we investigated by immunocytochemical techniques, the expression of these proteins in cells of the coverslip model. It was observed that cells on coverslips removed after 3, 5 and 7 days of implantation do not express these antigens. Nevertheless, cells previously characterized as epithelioid cells [64] on the surface of coverslips removed after 14 days of coverslip implantation are MRP-14$^-$/8$^-$ cells. We have also observed that epithelioid cells from typical (thread suture) granulomas also express MRP-14 but not MRP-8 antigens. These data show, contrary to the observations of DELABIE et al. [31], that epithelioid cells from foreign-body granulomata in man and mice express in their cytoplasm MRP-14 but not MRP-8 calcium-binding proteins. Taking these investigations further, we showed that MRP-14 calcium binding protein can be detected in the supernatant of epithelioid cell cultures and, that monoclonal antibodies anti-MRP-14 protein, blocks the MDF activity of this supernatant. Finally, highly purified MRP-14 but not MRP-8 blocks superoxide anion release by activated macrophages "in vitro". These results demonstrate a biochemical identity between MDF and the MRP-14 calcium-binding protein (MARIANO & SORG, *in preparation*). The mechanism of macrophage deactivation by MRP-14 is not yet determined. Nevertheless, activation of macrophages by lipid A immediately (30 sec.) causes a rapid and extensive intracellular fluxes of Ca^{++} i.e., the ion concentration rises from basal levels of 55 n*M* to ~ 600 n*M* [83]. Yet, inhibitors of calcium fluxes and protein synthesis respectively block macrophage activation by INF γ and LPS [47, 93]. Based on these observations, it is licit to speculate that MRP-14, a calcium binding protein of unknown function, in some way might down-regulate Ca^{++}

fluxes in activated macrophages thus deactivating these cells.

These results led us to hypothesize [62] that epithelioid cells secret the MRP-14 calcium-binding protein (MDF) which by deactivating the newly arrived cells impair antigen elimination with consequent persistence of the lesion.(Fig. 2)

If this hypothesis is true, it could be a plausible explanation for the persistence of tuberculous granulomas for instance, despite the existence of an effective and sterilizing peripheral immunity (Koch's phenomenon).

FINAL COMMENTS

Studies on the granulomatous inflammation with a variety of methods have provided a large amount of data which helped to elucidate intricate aspects of the phenomenon: first the granuloma is mainly composed of cells of the Mononuclear Phagocytic System, the epithelioid cells being the landmark of the process; second the granulomatous reaction is primarily a response of macrophages that is induced by certain types of "irritants"; third the lesion is maintained by a constant recruitment of cells from the circulation, mainly monocytes, which can be transformed into activated macrophages or epithelioid cells and which may occasionally fuse to form giant cells. Finally, lymphocyte responses may influence the lesion either to expand or to shrink. Nevertheless, many aspects of the granulomatous response remain to be elucidated. One of the most important questions to be solved concerns the characteristics of the granulomatogenic agents. Some but not all pathogens are kept alive within macrophages and induces these cells to aggregate which results in the typical morphology of the lesion. The epithelioid transformation of macrophages should be studied more in detail since this type of macrophage modulation is a unique manifestation of the process. A major limitation of these studies is the fact that the epithelioid modulation of macrophages has never been observed in vitro. The understanding of this phenomenon would certainly help to elucidate the role these cells play in granuloma formation, modulation and fate. Finally, once the interactions between the immune system and granuloma maintenance are better understood, prophylactic and chemotherapeutic agents shall be more efficiently developed to control a large spectrum of granulomatous diseases of man and animals.

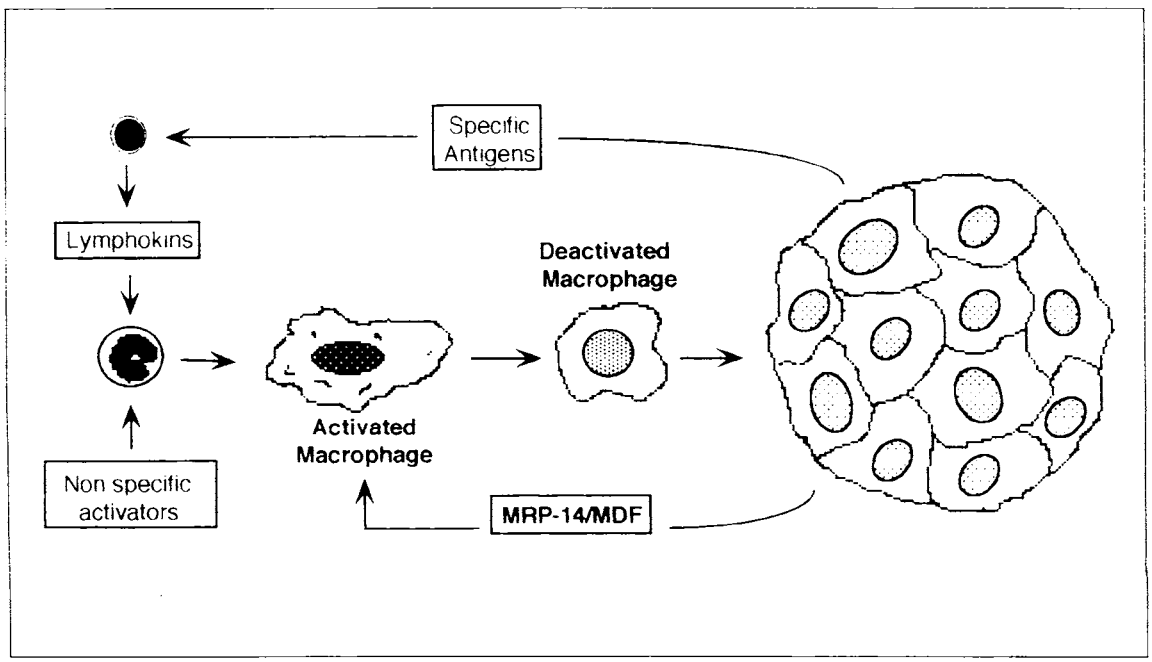

Fig. 2 - Schematic and hypothetical representation of the role played by MRP-14/MDF monokine secreted by epithelioid cells, on macrophage deactivation and consequent persistence of granulomatous lesions.

RESUMO

O granuloma experimental. Uma hipótese para explicar a persistência da lesão.

A inflamação glanulomatosa é o substrato morfológico de uma variedade de doenças infecciosas importantes tais como tuberculose, lepra, esquistossomose e outras. Embora muitos aspectos deste tipo especial de inflamação sejam conhecidos, questões fundamentais referentes à formação do granulona, persistência, destino e significado da relação hospedeiro-parasita permanecem para serem elucidados. Nesta revisão é apresentada a literatura básica e mais relevante relacionada à investigação experimental sobre a fisiopatologia do granuloma. Baseados em investigações recentes realizadas, em nosso laboratório mostrando que o fator de desativação do macrófago (MDF) secretado pelas células epitelióides e caracterizado pela proteina MRP-14, uma proteina ligante de cálcio, foi levantada hipótese para explicar a persistência da inflamação granulomatosa.

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