Immunopathogenesis of Pulmonary Granulomas in the Guinea Pig after Infection with *Mycobacterium tuberculosis*

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Pulmonary tuberculosis in guinea pigs is similar to the disease in humans and is accordingly widely used as a model to test tuberculosis vaccines. The primary site of expression of acquired immunity and the hallmark of tuberculosis is the granuloma. Granuloma morphology is well described, but there is limited information regarding T-cell subset influx. We monitored the course of pulmonary tuberculosis in guinea pigs and observed four distinct immunohistopathological stages. In all stages there were similar numbers and arrangement of CD4 and CD8 T cells. There were only small numbers of apoptotic lymphocytes, scattered around and within the necrotic core, and acid-fast bacilli were visible both within macrophages and free within airway debris. A key finding of the study was the observation that the development of the necrotic core was an early event and almost certainly preceded the emergence of the acquired immune response. This in turn suggests that innate mechanisms are the basis of the early lesions and that subsequent acquired responses are unable to moderate them. This hypothesis differs from the current dogma that excessive activity of T cells mediates delayed-type hypersensitivity and that cellular cytolysis is the root cause of the necrosis.

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*Mycobacterium tuberculosis* continues to kill approximately 2 million people per year (12), more than any other single human pathogen. In addition, it is estimated that in excess of 100 million people may carry the organism in a dormant or latent state (14). This has prompted a concerted effort to develop new vaccines with which to prevent the disease in its various forms (31, 33).

There are now several different animal models available with which to test new vaccines, ranging (in size and expense) from the mouse to primate models. It is widely accepted that perhaps the best model is the guinea pig (*Cavia porcellus*), given the similarities of this model to humans infected with tuberculosis in terms of the formation of the lung granuloma and its subsequent necrosis and mineralization (13, 18, 24, 37).

The formation of the lung granuloma is the classical hallmark of pulmonary tuberculosis. The morphology of the granuloma is characterized by a caseous necrotic core which is surrounded by a layer of epithelioid macrophages, which, in turn, is surrounded by concentric layers of lymphocytes and fibrosis (6). Multinucleated giant cells and occasional plasma cells are also seen. The influx of leukocytes and fibroblasts forming these structures is an attempt to segregate the infection, to prevent bacterial dissemination to the remainder of the lung and other organs, and to focus the immune response directly at the site of implantation. Its development involves an elaborate production and interaction of chemokines and cytokines by effector cells as well as local tissue cells. In addition, the upregulation of receptors for these molecules as well as for adhesion and integrin molecules on responder cells facilitates the proper coordination of the recruitment and migration of cells into the granuloma and their retention in the granuloma (39).

Because of the significant ways in which it resembles both the normal human physiology and the pathophysiology of pulmonary tuberculosis, the guinea pig is one of the more extensively studied animal models of tuberculosis (40). Following exposure to a small number of bacilli delivered via aerosol, the infection in the guinea pig lung grows progressively for about 30 days (41) before being contained by the host response (30). Similarly to other animal models however, this expression of host immunity is not completely effective, and as a result, many bacilli survive, giving rise to a chronic state of disease.

Although it has long been realized that the protective process in the guinea pig lung involves the formation of granulomas, there is surprisingly little further description of this event. For example, previous descriptions have characterized “primary” and “secondary” lesions with reference to only the size and amount of necrosis in the lesions (42). The guinea pig is well known to be a susceptible animal model that develops granulomas with prominent central caseation and extensive connective tissue deposition, yet knowledge of the arrangement of these elements over time remains obscure. In addition, the influx of T-cell subsets, only recently described in the mouse model (16), has yet to have been documented in the guinea pig.

Given the importance of the model to current vaccine development work, the purpose of this study was to chronicle the temporal and spatial arrangement of the cell influx, as well as other aspects of the overall process, and to attempt to relate these data to the known pathological process. The findings presented seriously challenge the current dogma that excessive expression of the acquired T-cell response triggers the pathologic degeneration of the lung granuloma.
MATERIALS AND METHODS

Animals. Female Hartley guinea pigs were obtained from Charles River Laboratories (Willington, Mass.). They were housed under biosafety level III conditions at Colorado State University and fed standard guinea pig chow and water ad libitum. After infection, the animals were assessed using a modified Karnofsky scale (20) for pain and distress in guinea pigs.

Bacterial infections. M. tuberculosis Erdman (TMCC 107) was previously grown to early mid-log phase from low-passage seed lots in Proskauer-Beck liquid medium containing 0.05% Tween 80. Cultures were aliquoted into 1-ml tubes and frozen at −70°C until used. Thawed aliquots were diluted in double-distilled sterile water to the desired inoculum concentrations. Guinea pigs were infected via the aerosol route with a low dose of bacteria. Briefly, the nebulizer compartment of a Middlebrook airborne-infection apparatus (Glas-Col, Terre Haute, Ind.) was filled with 5 ml of distilled water containing a suspension of bacteria known to deliver approximately 20 to 50 bacteria into the lungs.

Enumeration of bacteria. Groups of two guinea pigs were killed humanely at 11, 21, 31, 71, and 93 days after aerosol infection with M. tuberculosis strain Erdman. Only one animal survived to and was examined at 99 days postinfection. At necropsy, all lung lobes were removed from the thorax individually to enable separate manipulations with each lobe. The number of viable bacteria in the lungs was monitored over time by plating serial 10-fold dilutions of right cranial lung lobe homogenates onto nutrient Middlebrook 7H11 agar. The bacterial colony formations were counted after 21 days of incubation at 37°C under 5% CO2. The data were expressed as the log10 of the mean number of bacteria recovered.

Histological testing. The right middle lung lobe was first slowly infused through the major vessels at the hilus with 10% neutral buffered formalin. This was to prevent alveolar collapse and assist in morpometry. The lobe was then submerged in the formalin. After a minimum of 48 h, the tissue was prepared and sectioned for light microscopy, with lobe orientation designed to allow the maximum surface area to be seen. Consecutive sections were stained with hematoxylin and eosin and with Ziehl-Neelsen stain for the detection of acid-fast bacilli. Sections were also stained by the Masson trichrome method for the detection of collagen and by the Fraser-Lendrum method for the detection of fibrin (23). Sections were examined by a veterinary pathologist who had no prior knowledge of the experimental groups and were evaluated at least twice to verify the reproducibility of the observations.

Immunohistochemistry. Mouse anti-guinea pig monoclonal antibodies specific for guinea pig CD4 (clone CT7) and CD8 (clone CT6) were purchased from Serotec (Oxford, England). In each case, the Serotec mouse immunoglobulin G1 (IgG1)-negative control (clone W3/25) was used as the isotype control. F(ab′)2 rabbit anti-mouse Ig-globulin, also from Serotec was used as the secondary antibody. At necropsy, the left cranial lung lobe was first slowly infused with a 20% OCT (Tissue-Tek, Inc. Torrance, Calif.) and primary antibody for 30 min. The sections were then placed in a tissue mold, completely surrounded by OCT, frozen in a bath of liquid nitrogen, and stored at −70°C until used. Serial sections from each lung, 5 μm thick, were cut in a cryostome (CM 1850; Leica, Bannockburn, Ill.) by employing the Instrumentics Inc. (Hackensack, N.J.) tape transfer system, fixed in cold acetone for 5 to 10 min, and then air dried. Next, the sections were washed in APK buffer solution (Vetana Medical Systems, Tucson, Ariz.) for 15 to 20 min and incubated in a 1:50 (CD4) or 1:100 (CD8) solution of Protein Block goat serum (Biogenex, San Ramon, Calif.) and primary antibody for 30 min. The sections were then placed on a Nexus automated immunostainer (Ventana Medical Systems). The labeled avidin-alkaline phosphatase and Fast red/napthol detection kit was employed. The secondary antibody was incubated for 30 min at room temperature. Sections were counterstained with Meyer’s hematoxylin. Sections of spleen were also examined to act as positive controls.

Detection of apoptosis. The ApopTag Plus peroxidase in situ apoptosis detection kit (Intergen, Purchase, N.Y.) was used to detect apoptotic cells in paraffin-embedded tissue. Two or three 5-μm sections of the same tissue used for histological analysis were analyzed per time point.

Photomicroscopy and morphometry. Photomicroscopy was performed with an Olympus AH-2 microscope linked to a Sony SCK-DKS digital camera and Adobe Photoshop 6.0 software. In an attempt to further classify the pulmonary lesions, the granuloma fraction was calculated from each of the hematoxylin-and-eosin-stained histological slides. In brief, using Metamorph software (Metamorph software, Universal Imaging Corp.), the perceived inflammatory area was determined by outlining the affected tissue on the captured images and expressing this as a percentage of the total area of the observed lung tissue.

FIG. 1. Course of infection in guinea pigs exposed to aerosol infection with M. tuberculosis. At the indicated time points, the right cranial lung lobe was removed for enumeration of bacterial numbers. Data are expressed as the mean number of viable bacteria (CFU) and the standard error of the mean (where error is greater than 0.1).

RESULTS

Course of M. tuberculosis infection in the lungs of infected guinea pigs. The course of the infection after aerosol exposure is shown in Fig. 1. As anticipated, the infection increased progressively for the first 3 weeks and then stabilized into an apparent chronic state of disease. In the later stages of the experiment, there was evidence of an increase in the bacterial load, consistent with the worsening pathological condition described below.

Distinct cell populations are seen in guinea pig lung granulomas after aerosol infection with M. tuberculosis. The lesions that formed in response to the M. tuberculosis infection over time could generally be resolved into four histological stages, which are summarized in Table 1. Examples of each stage are shown in Fig. 2. Detailed descriptions of the four stages are listed below.

(i) Stage 1. By 11 days after infection, small (up to approximately 500 μm in diameter), discrete, round lesions composed of tight accumulations of epithelioid macrophages admixed with granulocytes could be seen (Fig. 2A and B). These cells have distinct eosinophilic cytoplasmic granules and are sometimes referred to as pseudoeosinophils (28). Similar numbers of lymphocytes were also present. The lesions were most commonly seen in the parenchyma, close to major airways and blood vessels, effacing and expanding alveolar septa.
FIG. 2. Representative photomicrographs of the four stages of pulmonary granuloma development. (A) Stage 1 granuloma (day 11). A single discrete round focus of macrophages admixed with granulocytes and small numbers of lymphocytes adjacent to a large blood vessel and airway. a, artery; b, bronchiole; ca, cartilage. Bar, 100 μm. (B) Stage 1 granuloma; area delineated by the black rectangle in panel A. Note the abundance of granulocytes (arrows) intermixed with epithelioid macrophages and the lack of normal alveolar structure. Bar, 10 μm. (C) Stage 2 granuloma (day 21). A single discrete round focus of macrophages with an eosinophilic necrotic core (c) abutting a bronchiole (b). Bar, 100 μm. (D) Stage 2 granuloma; area delineated by the black rectangle in panel C. Note the abundance of neutrophils (arrows) intermixed with epithelioid macrophages and lymphocytes. Karyorrhectic debris is present in and around the core (c), and there are multiple small scattered islands of fibrin (*). Bar, 10 μm. (E) Stage 3 granuloma (day 31). A discrete round lesion with a distinct necrotic core (c) is surrounded by a layer of epithelioid and...
TABLE 1. Four-stage categorization of the pulmonary lesions in infected guinea pigs

<table>
<thead>
<tr>
<th>Lesion stage</th>
<th>Extent of lesions (% GF)a</th>
<th>Macrophage types</th>
<th>Lymphocyte organization</th>
<th>Granulocyte localization</th>
<th>Stage sequelae</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Single (11: 0.1, 0.1)</td>
<td>Histiocytes, epithelioid</td>
<td>Few, scattered</td>
<td>Prominent, scattered</td>
<td>Alveolitis</td>
</tr>
<tr>
<td>2</td>
<td>Multifocal (21: 4.9, 19.1; 31: 7.1, 16.7)</td>
<td>Histiocytes, epithelioid</td>
<td>Few, scattered, with small aggregates at margin</td>
<td>Prominent, scattered around and in the core</td>
<td>Karyorrhectic debris, fibrin deposition in the core and surrounding mantle; fibroplasia; granulomatous lymphadenitis</td>
</tr>
<tr>
<td>3</td>
<td>Multifocal (71: 45.7, 51.8)</td>
<td>Epithelioid, foamy</td>
<td>Scattered plus a prominent halo surrounding macrophages</td>
<td>Prominent, scattered around and in the core</td>
<td>“Classical” granuloma; necrosis and fibroplasia; granulomatous lymphadenitis</td>
</tr>
<tr>
<td>4</td>
<td>Coalescing (93: 42.9, 52.1; 99: 56.1)</td>
<td>Epithelioid, foamy</td>
<td>Scattered aggregates or individual cells</td>
<td>Prominent, scattered throughout macrophage focus, around and in the core, and amid airway debris</td>
<td>Necrosis, mineralization, marked fibroplasia; multinucleated giant cells; airway epithelium erosion and purulent airway exudate; granulomatous lymphadenitis</td>
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a Hematoxylin-and-eosin-stained lung sections were examined by standard light microscopy, and several parameters of the lesions were characterized. The parameters included morphometric assessment of the extent of the pulmonary involvement (granuloma fraction), the predominant macrophage types present, and the overall arrangement of lymphocytes and neutrophils in the lesions. Several sequelae of each stage were also noted in the infected lungs. Each lesion in a section was characterized individually, and stages were defined which encompassed the different types of lesions that were present.

b Bold values represent days postinfection. Values following these are percent granuloma fractions (GF) from individual animals.

tous lymphadenitis was present with increasing prominence from this stage on.

(iii) Stage 3. By day 31, the lesion had taken the form of the “classical granuloma,” with a diameter of up to 2 mm (Fig. 2E and F). There was a prominent central necrotic eosinophilic core, which was packed with karyorrhectic debris. The core was surrounded by epithelioid macrophages and neutrophils, and lymphocytes were also present. Beyond this, the lesion contained a prominent collar of lymphocytes admixed with cords of fibrous connective tissue.

(iv) Stage 4. Stage 4 lesions (Fig. 2G and H) represented the end stage spectrum of granuloma evolution (the example shown is from day 93). There was extensive coalescence of multiple granulomas into a dense sheet of inflammation, often effacing more than half of the lung sections. The necrotic cores were typically mineralized and surrounded by epithelioid macrophages. An elaborate fibrous web that had enveloped large foamy macrophages marked the previous outer margin of the granulomas. Neutrophils and lymphocytes were randomly spread at all levels of the inflammation. A few multinucleated giant cells were scattered throughout the lesions. Acid-fast bacilli were often detected both within the macrophages effacing the lung parenchyma and free within the supplicative airway debris (data not shown).

Localization of lymphocyte subsets by immunohistochemistry. The distribution of CD4+ and CD8+ T cells in the developing lesions is shown in Fig. 3. On day 11, small numbers of lymphocytes were evident. There were apparently similar numbers of CD4+ and CD8+ cells, and there was no evidence of cellular aggregation. This pattern was similar on days 21 and 31, as the lesions considerably increased in size. As the lesions degenerated, mineralized lymphocytes could still be detected but were present in smaller numbers.

Characterization of the fibrotic response. The development of lesion fibrosis was monitored by using specific staining methods (data not shown). No response was seen until day 21, when small discrete regions peripheral to the core and extending toward the outer margin of the granuloma stained positive for fibrin, indicating vascular damage. Collagen was also detected forming irregular cords around cells adjacent to the core. By day 31, the prominent lymphocyte collar now present was interlaced by bands of collagen of variable thickness whereas foamy macrophages. This in turn is surrounded by a prominent basophilic layer of lymphocytes (+), which are admixed with macrophages at the extreme margins of the lesion. A bronchiole (b) is present at one margin. Bar, 100 μm. (F) Stage 3 granuloma; area delineated by the black rectangle in panel E. Note the karyorrhectic debris in and around the core area to the right (c), the dense sheet of epithelioid and foamy macrophages infiltrated by neutrophils surrounding it, and (on the left) a region rich in lymphocytes, between which there is considerable collagen deposition (arrow). Bar, 10 μm. (G) Stage 4 granuloma; area of the lesion showing a longitudinal section of a bronchiole (b) which is blocked with neutrophilic and necrotic debris (arrows) filling the lumen. Dense sheets of macrophages with scattered lymphocytes and interweaving fibrosis (f) surround the airway. Bar, 100 μm. All sections are stained with hematoxylin and eosin.
FIG. 3. Representative photomicrographs showing immunohistochemical staining for CD4⁺ (left panels) and CD8⁺ (right panels) T cells within the four stages of pulmonary granuloma formation: stage 1 (A and B), stage 2 (C and D), stage 3 (E and F), and stage 4 (G and H). For the top four panels, the scale bar represents 10 μm; for the bottom four panels the scale bar represents 100 μm. c, necrotic core. Arrows indicate individual positive cells. Labeled avidin-alkaline phosphatase with the naphthol red method was used for all sections, and hematoxylin counterstain was used. CD4⁺ T cells were stained with Serotec clone CT7, and CD8⁺ T cells were stained with Serotec clone CT6. Biotin-labeled F(ab')₂ rabbit anti-mouse Ig was used as the secondary antibody.
fibrin deposits were spread throughout the granulomas, as well as prominently focused around the core.

By day 93, the structure was dominated by thick bands of collagen deposition, forming a thick web that appeared to trap large numbers of foamy macrophages in addition to completely surrounding the mineralized core (Fig. 2G). In contrast, fibrin deposition was rarely seen at this stage.

**Distribution of apoptotic cells.** It has been suggested that apoptosis of infected cells may be a major defense mechanism against *M. tuberculosis*. As shown in Fig. 4, very few apoptotic cells could be detected at any of the four stages of the disease process. On days 11 through 31, some apoptotic cells could be seen close to the developing core lesion, but later during the course of the infection, only a few cells stained positive for apoptosis and appeared to be randomly distributed.

**DISCUSSION**

The guinea pig is widely considered the consummate animal model of human tuberculosis, given the similarities in the pathological responses to pulmonary infection; as a result, it is an important tool in the search for new vaccines. The results shown here are the first attempt to provide a comprehensive description of the stages of the disease in concert with immunohistochemical analysis documenting the influx of CD4+ and CD8+ T cells into the developing lesions. In several subse-

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**FIG. 4.** Representative photomicrographs of the distribution of apoptotic cells within the four stages of pulmonary granuloma formation. (A) Stage 1 granuloma. A few scattered single apoptotic cells are indicated by the arrows. Bar, 10 μm. (B) Stage 2 granuloma. Bar, 100 μm. (C) Stage 3 granuloma. At this time point, most apoptotic cells tended to be adjacent to the core (c). Bar, 100 μm. (D) Stage 4 granuloma. The arrowheads point to mineral debris at the margin of the core. Bar, 10 μm. All sections were stained by the Apo-Tag TUNEL method, using diaminobenzidine as the chromogen and methyl green as the counterstain.
quent experiments, it was shown that this progression was evident and reproducible. The results of these studies raise a variety of issues that challenge the current dogma.

The current dogma to explain the pathological process in the guinea pig and rabbit is based on the historic studies by Lurie and more recently by Dannenberg (8–11). In that model, it is held that the initial presence of the bacilli causes no detectable tissue damage at first. Then, as cell-mediated immunity is triggered, “excessive delayed-type hypersensitivity” mediating the cellular influx and inflammation, in concert with cytolytic T-cell activity, leads to the necrotic degeneration of the center of the lesion. It was further proposed that the development of this caseous necrosis initially limits the growth of extracellular bacteria, until (in the rabbit) the lesion liquefies and the animal dies.

In this paper we propose a new hypothesis, which differs considerably from the Lurie-Dannenberg model. It is apparent to us that there is a very rapid response to the infection despite the very low dose, and hence it is highly probable that this response is innate in nature. Given the preponderance of macrophages, which are already present in significant numbers in the lesion by day 10, this may involve CD1- or Toll receptor-mediated mechanisms (3, 5, 25, 26, 35, 46). In addition, however, there are also obvious and prominent pockets of granulocytes; these may represent a double-edged sword in that they may be contributing to early protection, as suggested previously (1, 34), but their accumulation in response to local tissue damage and their own subsequent degranulation (perhaps merely due to their short life span) may also contribute to the local pathological process. Given the presence of these eosinophil-like cells throughout the early response, the data suggests that these cells are continuously accumulating in the lesions over this period.

It is possible that this innate immune response, which happens very early during the course of the infection, may be the trigger to the initial development of the characteristic central necrotic core. This structure is already very obvious by day 21, which seems to indicate that it has begun development long before the emergence of the acquired response. This latter response seems to peak about 10 days later and is associated with a large number of lymphocytes (a mixture of CD4+ and CD8+ cells) entering the lesion and forming a layer of cells peripheral to the central core. Moreover, in this region there are multiple scattered areas of fibrin deposition, which are suggestive of vascular permeability, which in turn would create local hypoxic conditions and cause the lesion to further increase in size. In contrast, there was no evidence for the hypothesis that the central core was promoted by cytolytic T cells, since there were no aggregates of either CD4+ or CD8+ cells adjacent to the lesion. Accordingly, our new hypothesis suggests that these very early mechanisms of innate immunity resulted in a process of irreversible damage that the subsequent emergence of the acquired response was too slow to prevent.

In a sense, moreover, the period of chronic disease seen between about days 31 and 71 might also be controlled in part by innate mechanisms. There is no evidence that the process of mineralization has an immune basis, and the substantial fibrotic response could also be regarded as a primitive event designed to wall off the lesion. Unfortunately, however, this process of consolidation is itself damaging, allowing erosion of cellular debris (including extracellular bacteria) into surrounding airways.

These guinea pigs succumb to the disease at about 100 to 140 days after exposure. We propose that at least two mechanisms underlie this event. First, the size of the lesion consolidates the lung lobe and interferes with efficient gas exchange. Second, the dissemination of the infection via debris deposition in the airways results in fresh ingestion by monocytes and the subsequent triggering of gamma interferon production by memory T cells. As a result, activated macrophages secrete large quantities of tumor necrosis factor alpha, leading to the weight loss invariably seen for a week or so prior to death of the animal.

Both M. bovis BCG and several new candidate vaccines are highly protective in the guinea pig model, which seems to suggest that this animal is capable of generating a potent memory T-cell response (2, 19). This in turn explains the very rapid lymphocytic response seen in vaccinated animals, which would be needed to prevent the development of the central necrotic core. As one of us has suggested elsewhere (32), this appears to be an important parameter of effective vaccination in this animal model.

Another important finding here was the observation that very few lymphocytes stained positive for apoptosis, suggesting that this mechanism does not play significant role in the disease process. This finding is rather contrary to the bulk of the literature (7, 15, 17, 21, 22, 27, 29, 38, 47), which holds that apoptosis of both T cells and infected macrophages is an important defense mechanism, although we note that most of these observations were all made in vitro.

Finally, although the mouse and guinea pig are considered to be quite different models of tuberculosis, there are also some similarities. If one regards the course of the disease in the guinea pig as having a stage of chronic disease followed by reactivation, then the fact that the latter stage is predisposed by much earlier events has certain similarities to the situation in various inbred mouse strains that are also prone to this event (45). Moreover, both animal models show evidence of a very efficient fibrotic response leading to deposition of collagen to provide some degree of integrity to the granuloma (30, 36).

Where there appear to be some interesting differences are in terms of the lymphocyte response. We have previously pointed to differences in terms of the propensity of mouse lymphocytes to accumulate toward the center of the granuloma (16, 30), and it may be that lymphocytes in the guinea pig occupy a more peripheral position simply because of the presence of the central necrosis. We have described clear distinctions between the CD4 and CD8 responses in mice, with CD4+ cells forming large aggregations dominating the granulomas and with CD8+ cells being more sparse and distributed more toward the periphery of the lesion (16). These data, when viewed with our data on CD8 gene knockout mice (43), are consistent with an immunosurveillance role for the CD8 population.

In the guinea pig, the distributions of the two T-cell subsets seem to be very different. Although large numbers of lymphocytes entered the lesions by day 31, they remained a fairly even randomized mixture of CD4+ and CD8+ cells, with no evidence of any cellular aggregation. In addition, they did not appear to collectively represent the entire population of lymphocytes, suggesting that the additional cells might be B cells and other cells, as previously seen in the mouse model (4, 16, 44). Whether this implies that CD8+ cells play a more prominent role in the early protective response in the guinea pig than in the mouse is impossible to say at this point.
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