Experimental Immunogenic Granuloma of the Orbit: Transfer of Granulomatous Hypersensitivity With a Subset of T Lymphocytes

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An experimental model to investigate orbital granuloma formation in inbred rats was established. Animals sensitized to trinitrophenyl ovalbumin (TNP-OA) and challenged retro-orbitally with TNP-OA covalently linked to Sepharose 4B beads specifically developed a granulomatous response. This granulomatous reactivity was passively transferred into normal animals by lymph node cells, but not by serum antibody from sensitized donors. Lymphocytes which transfer granuloma formation in normal recipients were characterized by cell fractionation and membrane marker analysis. These experiments show that the effector cells capable of transferring granulomatous hypersensitivity are enriched in the lower density fractions on discontinuous Percoll gradients. These cells are lymphoblasts and express the W3/25 helper T lymphocyte marker. It was also demonstrated that lymphoid cells from sensitized donors in the higher density Percoll fraction appear to be incapable of adoptively transferring granulomatous responsiveness directly to normal recipients. However, incubation of these high density lymphocytes with specific antigen resulted in marked enhancement of their ability to transfer the disease. Antigen-induced activation also resulted in an increase in both lymphoblasts and the W3/25 marker. The authors conclude, therefore, that a subset of T cells which are lymphoblasts and express the helper-cell marker is responsible for granuloma formation in sensitized animals and is capable of transferring orbital granuloma formation to non-sensitized normal recipients. Invest Ophthalmol Vis Sci 27:70-76, 1986

Pseudotumors of the orbit are characterized by concentrated aggregates of chronic inflammatory cells, whose cytology may fall anywhere on the spectrum from purely lymphoid hyperplasia to granuloma formation. Our current understanding of idiopathic inflammatory pseudotumors rests solely on numerous clinical and histopathologic reports (reviewed in ref. 1), but information on the etiology and pathogenesis of this disease is still lacking. It has been shown in several laboratories, however, that chronic immunogenic inflammatory reactions, with lymphoproliferative and granulomatous complications, may be induced in a variety of extraocular sites.2-5 Using a similar approach, we reported earlier the development of an interesting experimental model of orbital granuloma formation in the guinea pig.6 These immunogenic inflammatory responses were induced in animals sensitized to bovine gamma globulin (BGG) after retropalbar challenge with BGG, made insoluble by covalent coupling to Sepharose beads. In the experiments described below, we have confirmed and extended the earlier findings by adapting this animal model of orbital pseudotumor to the inbred rat. This has permitted the identification of the subset of T lymphocytes which mediates these immunologically-induced granulomas, and is capable of transferring the lesion to antigen-challenged naive recipients.

Materials and Methods

Animals

Female Lewis rats weighing approximately 150 g were used throughout the experiments.

Preparation of Antigen-Coated Beads

The hapten-protein conjugate, trinitrophenyl ovalbumin (TNP-OA), was prepared by the method of Ritenberg and Amkraut.7 TNP-OA was covalently coupled to cyanogen bromide-activated Sepharose 4B beads, utilizing previously described methodology.6 In brief, Sepharose 4B beads (Pharmacia; Piscataway, NJ) were sieved through Spectramesh (Fisher; Springfield, NJ) to select in the range of 40-60 μm in diameter. A 0.2 g/ml slurry of the activated beads in 0.1 M sodium...
Immunization and Challenge

Twelve Lewis rats were sensitized by footpad injection of 25 μg of TNP-OA emulsified in complete Freund's adjuvant (CFA, Difco Lab.; Detroit, MI). Animals were challenged retro-orbitally with TNP-OA-coated beads in their right (test) eyes and with uncoupled beads in their left (control) eyes 12 days after sensitization. Nonsensitized rats were injected with TNP-OA-coated beads as additional controls of the effect of challenge. Each challenge was delivered in a volume of 10 μl, containing 25–30 beads and a total quantity of 2 μg of TNP-OA. Groups of 4 animals were killed at various times after the injection of beads.

Histologic Analysis of Inflammatory Responses

The eyes were enucleated, fixed in 10% phosphate-buffered formalin, processed by standard histologic methods, and stained with hematoxylin and eosin. Histologic sections were made of ten different areas of the whole eye, and the inflammatory lesions were evaluated as follows: a negative response was scored when the test sections showed no more than a minimal foreign body reaction around the beads at 2–4 days than was shown by the controls; a positive reaction was scored when the varying degrees of chronic inflammatory infiltrate, composed of lymphocytes, macrophages, and epithelioid and giant cells, surrounded the beads and extended into the neighboring parenchyma.

Passive Transfer Experiments

All peripheral lymph nodes were harvested and pooled from twelve donor rats 12 days after immunization with TNP-OA in CFA. The state of delayed hypersensitivity was found to be strongly positive 12 days after sensitization by skin reaction with 2 μg of TNP-OA. Control donor animals were immunized only with CFA. Cell suspensions were prepared by gentle teasing, washed twice with Hanks' balanced salt solution (HBSS), adjusted to the desired concentration, and injected intravenously into four naive Lewis recipients. Each recipient received a quantity of cells approximately equivalent to the cellular content of three donor lymph nodes. In some experiments, groups of normal rats were injected intraperitoneally with 10 ml of pooled sera obtained from experimental and control animals. Antibodies to the TNP determinant were measured by the quantitative precipitin test. In these and all subsequent passive transfer experiments, challenge with TNP-OA coated beads was made retro-orbitally 24 hr later, and the animals were killed 3 days later for histologic examination.

Enrichment of T and B Cells

Lymph nodes from normal or sensitized donors were removed, made into single-cell suspension, incubated with glass beads, and separated into adherent (B cell-enriched) and nonadherent (T cell-enriched) populations according to the method of Neilson and Phillips. Briefly, 5 × 10^7 cells in 15 ml of RPMI-1640 with 5% fetal bovine serum (FBS) were added to a 75-cm² Corning tissue culture flask containing 6 ml of sterilized glass beads (type 100–500μ; 3M Company, St. Paul, MN). The flasks were incubated for 1 hr at 37°C. The nonadherent cells were resuspended by swirling, and removed. The flasks were then washed with cold HBSS without calcium and magnesium to remove adherent cells. After separation, the cells were washed, resuspended in HBSS at the desired concentration (10 × 10^7 cells per rat), and injected iv into normal recipients.

Discontinuous Percoll Gradient Centrifugation

Peripheral lymph node cells obtained from immune donors were pooled and depleted of adherent cells by incubation with glass beads. The nonadherent cells were fractionated on discontinuous Percoll gradients as described by Kurnick et al. Then 8 × 10^7 cells were pelleted in a 15-ml sterile conical centrifuge tube with screw cap (Corning; Corning, NY), and resuspended in 4 ml of the most dense (1.090 gm/cm³) Percoll solution. This was overlayed with 4 ml of less dense solution (1.077 gm/cm³). The gradients were then layered with 4 ml of HBSS. The tubes were centrifuged at 450 g for 15 min at room temperature. The cells were carefully collected by aspiration from each interface beginning at the top of the gradient, and each population was washed three times with HBSS, counted, resuspended in HBSS at the appropriate cell concentration, and injected iv into naive recipients.

Cell Culture

Because antigen-specific activation of primed T lymphocytes requires the participation of accessory cells, peritoneal macrophages from normal rats were allowed to adhere to the culture flasks. Peritoneal exudate cells were obtained from normal Lewis rats 4 days after i.p. injection of 10 ml sterile AMPAK (American Quinine; Shirley, NY). These cells were
plated at $10^6$ cells/ml of RPMI 1640 with 10% FBS and incubated for 5 hr at 37°C. After 5-hr incubation, the nonadherent cells were removed, and the adherent cells were washed three times with the medium. The cells of normal or immunized rats, obtained from floating on 70% Percoll (density = 1.090 gm/cm³), were added to the macrophage-attached culture flasks, and co-cultured at $2 \times 10^6$ cells/ml with or without TNP-OA (2 μg/ml) for 3 days at 37°C in a 5% CO₂ cell incubator. The culture medium was RPMI 1640 supplemented with 2 mM L-glutamine, 100 units penicillin, 100 μg streptomycin, 5% heat-inactivated FBS, and 2 $\times 10^{-3}$ M 2-mercaptoethanol. After 3 days culture, the cells were washed, resuspended in HBSS, and injected iv into normal syngeneic recipients.

T and B Cell Determination

Murine monoclonal antibodies specific for rat T lymphocytes were purchased from Accurate Chemical and Scientific Corp.; Hicksville, NY: W3/13, specific for all rat peripheral T cells; W3/25, specific for T helper cells; and OX-8, specific for the T suppressor/killer subset. $1 \times 10^6$ cells in 50 μl HBSS containing 0.02% NaN₃ were incubated for 30 min at 4°C with 10 μl of the appropriate monoclonal antibody. The cells were washed three times and labeled for 30 min at 4°C with 10 μl of fluoresceinated F(ab')₂ fragments of goat anti-mouse IgG (Cappel, Cochraneville, PA). Controls, substituting normal mouse IgG for the monoclonal reagents, yielded less than 1% nonspecifically stained viable cells. For surface Ig, $1 \times 10^6$ cells in 50 μl HBSS containing 0.02% NaN₃ were incubated for 30 min at 4°C with fluoresceinated F(ab')₂ fragments of rabbit anti-rat Ig with specificity for heavy and light chains (Cappel) and further processed as described above.

Assessment of Lymphoblasts

After separation of functional subpopulations, the cells were assayed for lymphocyte transformation in the absence of antigen. $1 \times 10^6$ cells were cultured in fresh medium without TNP-OA, and pulsed with 1 μCi $^3$H-thymidine (New England Nuclear; Boston, MA) for 12 hr at 37°C. A 10% trichloracetic acid precipitate from the cultured cells was collected on glass fiber filters (Whatman Inc., Clifton, NJ), and radioactivity measured in a Packard liquid scintillation counter (Packard Instruments; Downers Grove, IL). The cells were routinely assayed for size microscopically, to confirm the validity of the $^3$H-thymidine uptake as a measure of lymphoblasts.

These investigations conform to the ARVO Resolution on the Use of Animals in Research.

Results

Immunologic Specificity of the Granulomatous Reaction

The specificity and the kinetics of the granulomatous response to TNP-OA-coated beads were evaluated in Lewis rats sensitized with TNP-OA in CFA. Sensitized animals were challenged retrobulbarly with TNP-OA-coated beads in their right (test) eyes and with plain beads in their left (control) eyes 12 days after immunization. The orbital tissues were examined at 2, 4, and 8 days after injection of the beads. Four animals were tested at each time period. Beads were seen in the Harderian gland, muscle, and retrobulbar connective and adipose tissues. Since the Harderian gland occupies a relatively large space in the orbit of the rat, the beads lodged mainly in this tissue. Our studies of the guinea pig model, involving the use of protein antigens (BGG or OA), were complicated by the multiplicity of protein determinants. It seemed more advantageous in this rat model to induce granuloma formation to the small, better-characterized TNP haptenic determinant. In general, the distribution and extent of granulomatous inflammation around the beads paralleled those seen in the guinea pig model.

At 2 days, intense inflammatory infiltrates were seen around the TNP-OA-coated beads in the right eyes of the immunized animals (Figs. 1A-B). The beads were seen in small clusters, surrounded mainly by macrophages with variable numbers of lymphocytes, occasional multinucleated giant cells, and the rare cluster of polymorphonuclear cells. At 4 days, the lesions were smaller and the inflammatory cells more tightly packed, with the appearance of epithelioid cells; numerous multinucleated giant cells were also found. By 8 days after challenge, the inflammatory changes were minimal and proceeding toward healing, with minimal numbers of mononuclear and occasional giant cells adjacent to the beads. In contrast, the control left eyes of immunized animals challenged with uncoupled beads, and of non-immunized animals challenged with TNP-OA-coated beads, did not show comparable inflammatory responses at any time. The beads showed either no surrounding reaction, or only a very mild foreign-body response (Fig. 1C).

Passive Transfer Experiments

Passive transfer of granulomatous reactivity into normal recipients was performed by injection of lymph node cells or serum from sensitized animals. Four naive recipients were injected with either $3 \times 10^8$ immune lymph node cells or 10 ml of serum containing 0.5 mg/ml of anti-TNP antibodies. Twenty-four hours after cell or serum transfer, the recipients were challenged
by retrobulbar injection of TNP-OA-coated beads in the right (experimental) eyes and corresponding numbers of uncoated beads in the left (control) eyes. The animals were killed 3 days later, the time at which actively sensitized animals demonstrate the early development of granuloma formation. While the right eyes injected with TNP-OA-coated beads developed significant granulomatous inflammation in the orbital tissues, all the left eyes that received plain beads showed only minimal foreign-body reactions. In contrast, there was only mild inflammatory reaction consisting mainly of polymorphonuclear leukocytes around the TNP-OA-coated beads in the five animals that received anti-TNP antiserum. These experiments demonstrate that specifically sensitized lymphocytes, rather than antibodies, play the major pathogenic role in the formation of orbital granulomas.

Characterization of Effector Cells

The cells responsible for transferring granuloma formation to normal recipients were identified in the following manner. Suspensions of lymph node cells from normal or sensitized animals were subjected to a lymphocyte-enrichment procedure, utilizing the differential adherence of T and B cells to glass beads. The cell populations were assayed for their T and B cell content by immunofluorescent staining. As shown in Table 1, the nonadherent cells were primarily T cells, whereas the adherent cells were predominantly B cells. $1 \times 10^8$ adherent or nonadherent cells were injected iv into groups of five normal recipient rats. Twenty-four hours after cell transfer, the recipients were challenged by retrobulbar injection of TNP-OA-coated beads, and the orbital tissues examined histologically 3 days later. The TNP-OA-coated beads produced significant granulomatous inflammation in those recipients that had received nonadherent cells (T cells), whereas the animals that received adherent cells (B cells) showed only mild foreign-body reaction (Table 1). In contrast, the TNP-OA-coated beads induced either no reaction or only very mild foreign body inflammatory reactions in rats that received nonadherent cells obtained from normal, nonsensitized donors.

Further characterization of the nonadherent, T-cell rich populations that mediate the transfer of granuloma formation demonstrated that a particular subset of T cells is responsible for the inflammatory lesions. Since commercially available monoclonal anti-rat T cell antibodies are not cytotoxic in the presence of complement, negative selection for a particular T cell subset could not be done. Therefore, we employed a rapid method for the separation of lymphoid cells on the basis of differences in their density on discontinuous Percoll gradients. When the nonadherent cell populations were subfractionated on density gradients, cells floating at a density of 1.077 (designated low-density T cells) were found to transfer granulomatous responses to normal recipients, whereas cells at a density of 1.090
(designated high-density T cells) were inactive. Approximately 10% of the nonadherent cell populations applied to the gradient were recovered in the low-density fraction, and only relatively small numbers (2 \( \times 10^5 \)) of these low-density T cells were required to adoptively transfer granuloma formation to normal recipients.

Table 1. Adoptive transfer of granulomatous response with T lymphocytes*

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Number of cells transferred ((\times 10^3))</th>
<th>Incidence†</th>
<th>(^{3}HTdR) uptake</th>
<th>W3/13</th>
<th>W3/25</th>
<th>OX-8</th>
<th>Ig</th>
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<td>Adherent cells</td>
<td>10</td>
<td>0/5</td>
<td>1435</td>
<td>11.5</td>
<td>nd*</td>
<td>nd</td>
<td>60.2</td>
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<tr>
<td>Nonadherent cells</td>
<td>10</td>
<td>3/5</td>
<td>780</td>
<td>84.2</td>
<td>37.5</td>
<td>10.3</td>
<td>8.1</td>
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<td>Low-density T cells</td>
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<td>7167</td>
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<td>Culture without TNP-OA</td>
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<td>0/5</td>
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<td>59.4</td>
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<td>1123</td>
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* Recipients killed 3 days after retrobulbar injection of TNP-OA-coated beads. † Proportion of recipient rats with positive histology. * Not done.

in the presence of antigen to generate the capacity to transfer granuloma formation. When high-density T cells were cultured for 3 days in the presence of TNP-OA and normal peritoneal macrophages as accessory cells, three of five recipients of \( 5 \times 10^5 \) of the cultured cells developed granulomatous inflammation after challenge with TNP-OA-coated beads (Fig. 3 and Table 1). When these cultured cells were fractionated on discontinuous Percoll gradients, approximately 5% of the cells were now found in the low-density fraction and were enriched in W3/25 helper cells and blasts. On the other hand, high-density T cells cultured without TNP-OA were still inactive (0/5 cells), and very few cells could be recovered in the low-density fraction after culture. It is likely, therefore, that the high-density T cells contain antigen-specific memory T cells, which can be activated by in vitro culture with antigen.

Discussion

We have demonstrated in these studies that immunogenic granulomatous lesions can be successfully and consistently produced in the lacrimal gland and other orbital tissues of the inbred rat. The basic experimental design consists of the injection of insoluble beads coated with antigen into the orbital tissues of specifically sensitized animals. These lesions have many features in common with those seen in orbital pseudotumors. These beads induce chronic inflammatory reactions characteristic of granulomatous hypersensitivity. The granulomatous response induced is specific, because normal animals challenged with antigen-coated beads, or sensitized animals challenged with uncoupled beads, do not develop either substantial inflammatory infiltrates or granulomas.

Granulomatous reactivity can be transferred passively into normal animals by lymph node cells but not by serum antibody from the same sensitized donors. The cells responsible for the transfer of granuloma formation to normal recipients were identified by cell
fractionation and membrane marker analysis. Our experiments show that a low-density lymphoblastic subset of T cells bearing the W3/25 T helper marker is responsible for the passive transfer of granuloma formation to naive recipients and thus, presumably, for the lesions induced in sensitized animals.

Various experimental models have been used to study the relationship of granuloma formation to cell-mediated immunity. These studies have employed a variety of soluble antigens derived from mycobacteria, fungi, and protozoa as well as bland protein antigens (reviewed in ref. 11). All of these results point to the essential role of cell-mediated immunity in the pathogenesis of experimentally induced granulomas. More recent studies have examined the role of T lymphocytes in initiating and modulating delayed hypersensitivity granuloma formation.5,12 The effector T cells which transfer granulomatous hypersensitivity in mice express the Lyt-1+2,3- membrane alloantigen.12 The W3/25+ rat T cells appear to be similar to mouse Lyt-1+2,3- lymphocytes.13 Our work is thus in agreement with results of Wellhausen et al., since passive transfer of granulomatous disease is obtained in the rat with T lymphocytes bearing the W3/25+ helper cell marker.

With respect to the mechanism of the transition from a lymphoproliferative to a granulomatous inflammation, it is likely that the interaction of locally retained antigen (antigen-coated beads) and sensitized helper T cells triggers these W3/25+ cells to release a variety of lymphokines. These, in their turn, recruit and activate additional lymphocytes and especially macrophages, contributing to granuloma formation in an accelerated manner. In recent years it has become evident that lymphokines play an important role in the initiation and maintenance of granulomas.14 Thus, Wellhausen et al.,12 have demonstrated that Lyt-1+2,3- cells responsible for transfer granuloma formation are identical with those which produce migration inhibition factor in Schistosome-infected mice. Furthermore, it has been demonstrated that the production of interleukin 2 is confined to Lyt-1+ T cells from murine spleen,15 and to low-density T lymphocytes from human peripheral blood.16 Taken together, these findings support the assumption that rat W3/25+ T cells, by analogy with the murine system, may initiate granuloma formation via lymphokine production.

In view of the functional tests, the assessment of lymphoblasts, and the marker analyses described in Table 1, we assume that the density differences among T cells represent cells in different stages of maturation (or differentiation). This is supported by our observa-

Fig. 2. A, Orbital tissues of a normal rat with adoptively transferred sensitivity. The recipient received $2 \times 10^7$ low-density T lymphocytes, was challenged by retrobulbar injection of TNP-OA-coated beads 24 hr after cell transfer, and was killed 3 days later. The response is similar to that seen in actively sensitized animals, Fig. 1A (H&E, ×240). B, Orbital tissues of a normal rat adoptively transferred with $2 \times 10^7$ high-density T lymphocytes, after three days. The inflammatory response is minimal (H&E, ×240).

Fig. 3. Orbital tissues of a normal rat sensitized by passive transfer of $5 \times 10^7$ high-density T lymphocytes after in vitro activation with antigen. A severe inflammatory reaction is present 3 days after challenge with coupled beads, demonstrating the activity of these stimulated cells (H&E, ×240).
tion that the larger T cells, which are found in the less dense fraction of the gradient and which express an increased density of W3/25 alloantigen, are responsible for the passive transfer of granulomatous reactivity. The smaller lymphocytes are found in the denser fraction, and express little W3/25. While ineffective in transferring sensitivity, they nevertheless have the potential to do so after in vitro culture (activation) with antigen, as shown in Table 1. This activation treatment increases their content of W3/25 marker and also their size.

The development of this experimental model of orbital granuloma, and the beginning elucidation of the cell types responsible for its formation, open the way to a further definition of the pathogenesis of these lesions and perhaps even to their prevention. Our findings suggest that one approach to the inhibition of granuloma formation may involve the suppression of the W3/25* subset of T cells, such as might be accomplished with specific anti-idiotype antibodies.\(^5\)\(^-\)\(^7\)

**Key words:** orbital granuloma, helper T cells, memory T cells, lymphocyte membrane marker, passive transfer

**References**