T Suppressor Cells as Well as Anti-Hapten and Anti-Idiotype B Lymphocytes Regulate Contact Sensitivity to Oxazolone in Mice Injected with Purified Protein Derivative from *Mycobacterium tuberculosis*

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Purified protein derivative from *Mycobacterium tuberculosis* inhibits contact sensitivity to oxazolone in mice when given intravenously 24 to 72 h before the antigen. Transfer experiments showed that various types of suppressor cells occurred in the lymph nodes draining the site of sensitization: (i) anti-oxazolone idiotype* B lymphocytes, found at day 3 after sensitization, transferred suppression to syngeneic recipients at the moment of their sensitization; (ii) anti-idiotype B lymphocytes, found at day 3 after sensitization, transferred suppression to syngeneic recipients when sensitization of these animals had been performed 3 days before cell transfer; (iii) T lymphocytes, found only at day 6 after sensitization, inhibited the passive transfer of contact sensitivity, indicating that they were effective on the effenter phase of the immune response. These results indicate that purified protein derivative from *M. tuberculosis* interferes with contact sensitivity by activating a complex and multiple immunoregulatory circuit.

Many microorganisms or bacterial constituents are polyclonal B-cell activators (PBAs) (6, 10, 21, 26), in that they induce B lymphocytes to proliferate and mature into immunoglobulin-secreting cells. Under appropriate conditions, PBAs cause several alterations in immune reactivity, which include the replacement of the need for T cells in antibody formation to antigens which normally require cellular cooperation (13), the inhibition or enhancement of antibody production when given before or after the antigen, respectively (8, 20, 23), and the depression of cell-mediated immunity (12, 15, 19). Nonetheless, the mechanism of action of PBAs has not been well defined. For instance, the inhibition of the humoral immune response, induced by lipopolysaccharide given 24 to 72 h before the antigen, has been attributed to the capacity of lipopolysaccharide to induce maturation of virgin B cells into antibody-forming cells, thereby depleting the number of precursor cells available for subsequent stimulation by the antigen (20). However, such a mechanism could not account for the depression of cell-mediated immunity. Several studies, including our own, have shown that PBAs inhibit delayed-type hypersensitivity reactions through the activation of B lymphocytes (4, 6, 7, 11, 12). The present study was designed to investigate whether the purified protein derivative from *Mycobacterium tuberculosis* (PPD), another well-known PBA (26), affects delayed-type hypersensitivity evaluated in terms of contact sensitivity to oxazolone.

The results provide evidence that PPD inhibits contact sensitivity in mice by favoring the appearance of suppressor cells which are anti-oxazolone (anti-ox) idiotype-positive (Id') B lymphocytes, anti-idiotype (anti-Id) B lymphocytes, and T lymphocytes. However, these cells occur and exert their effects at various phases of the sensitization process. The role and the mechanism of action of these cells are discussed.

**MATERIALS AND METHODS**

**Animals.** Inbred C57BL/6 mice of both sexes, 8 to 12 weeks old, were used throughout this investigation. In each experiment, the animals were allocated at random to the various groups. Each group consisted of six to seven mice.

**PPD.** PPD was kindly provided by R. Vanni (Istituto Scavo, Siena, Italy). Mice were injected intravenously (i.v.) with 500 μg of PPD, unless otherwise stated. Control groups were injected i.v. with 500 μg of bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.).

**Sensitization and detection of contact sensitivity.** Mice were sensitized by painting the skin of the abdomen and lower thorax with 0.2 ml of 1.5% ethanolic oxazolone (4-ethoxy-2-methyl-2-phenylazolone) or 3% picryl chloride, both from BDH, Poole, United Kingdom. Sensitized mice were challenged 6 days later by painting their ears with a drop of 1% oxazolone or picryl chloride in olive oil according to the sensitization schedule. The quantification was made by measuring the increase in ear thickness with a micrometer 24 h later (1 unit = 10⁻³ cm).

**Oxazolone and picrylsulphonic acid coupling to HSA.** Oxazolone was coupled to human serum albumin (HSA) by the procedure described by Askenase and Asherson (3). HSA was conjugated with picrylsulphonic acid by a slight modification of the method of Rittenberg and Amkraut (24).

**Anti-ox Abs and anti-Pcl Abs.** Mice were sensitized by painting the skin of the abdomen and lower thorax with 0.2 ml of a solution of 1.5% oxazolone or 3% picryl chloride. The sensitization was repeated three times at weekly intervals. After 3 weeks, sera from sensitized mice were collected and pooled. Anti-ox and anti-picryl chloride antibodies (anti-Pcl Abs) were purified by affinity chromatography on antigen with the Sevates Insolmer Kit (Sera-Lab, Crawley Down, Sussex, United Kingdom) by the procedure instructions.

**Transfer of contact sensitivity.** Donor mice were killed 3 days after sensitization; the draining (brachial, axillary, and inguinal) lymph nodes (LN) were removed, dissociated in Eagle minimal essential medium (Wellcome Research Labo-
and sensitized cells from the solution; wool column glass (anti-Id, calf with with later, as with picryl chloride. The recipients were challenged 6 days later as described above.

Separation of T and B lymphocytes. Cells from the draining LN, teased in Eagle medium supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) and washed twice with the same medium, were filtered through a glass wool column to remove macrophages (14) and then filtered through a nylon wool column (29). This method yielded: (i) effluent lymphocytes with 95% viability, of which 87 to 96% were theta-bearing cells and (ii) nylon wool-adherent lymphocytes with 95% viability, of which 4 to 8% bore theta antigen and 75 to 85% carried membrane surface immunoglobulins. This second fraction contained macrophages which were generally fewer than 2% of the total count.

Anti-Thy 1.2 serum treatment. LN cells (2 x 10^7/ml) were mixed with an equal volume of a monoclonal anti-mouse Thy 1.2 serum (New England Nuclear, Dreieich, Federal Republic of Germany) diluted 1:100 in Eagle medium and held on ice for 45 min. After being washed, the cells were mixed with an equal volume of fresh guinea pig complement diluted 1:5 in Eagle medium and incubated for 45 min at 37°C. This treatment killed 39 to 48% of the LN cells as judged by nigosine dye exclusion.

Anti-mouse immunoglobulin serum treatment. To remove B lymphocytes, LN cells were treated with a 1:10 dilution of rabbit anti-mouse immunoglobulin serum (Cappel Laboratories, Cochranville, Pa.) and complement by the same procedure as described above. This treatment killed 29 to 34% of the cells.

B-cell separation. "Panning" experiments were performed by the method described by Wysocki and Sato (31) with some modifications. Briefly, tissue culture petri dishes (9-cm diameter) were coated with oxazolonated or picrylated HSA at 200 µg/ml or with purified anti-ox or anti-Pcl Abs. After overnight incubation at 4°C, the fluids were removed and saved for reuse. The plates were washed three times and neutralized with medium supplemented with 40% fetal calf serum for 30 min to limit nonspecific adherence. Panning was at 3.5 x 10^3 live cells in 3.5 ml of medium supplemented with 10% fetal calf serum. The enriched B cells obtained after nylon wool treatment were applied for 1 h at 4°C to petri dishes coated with oxazolonated or picrylated HSA or coated with purified anti-ox or anti-Pcl Abs. After incubation, the plates were shaken and the cells were poured off: both the anti-ox depleted B-cell subpopulation, obtained from the plates coated with oxazolonated HSA, and the anti-Id depleted B-cell subpopulation, recovered from the plates coated with anti-ox Abs, corresponded to 45 to 50% of the original B cells. To recover the bound cells, the plates were filled with phosphate-buffered saline supplemented with 5% fetal calf serum, and the entire surface of the plates was flushed by using a Pasteur pipette. Oxazolone-bound B cells (anti-ox, B-cell-enriched subpopulation), obtained from the plates coated with oxazolonated HSA, represented 16 to 20% of the original total, whereas anti-ox Abs-bound B cells (anti-Id, B-cell-enriched subpopulation), recovered from the plates coated with anti-ox Abs, were 20 to 28% of the original total. Pcl-bound B cells (anti-Pcl, B-cell-enriched subpopulation), obtained from the plates coated with picrylated HSA, corresponded to 12 to 15%, and anti-Pcl Abs-bound B cells (anti-Id, anti-Pcl, B-cell-enriched subpopulation), recovered from the plates coated with anti-Pcl Abs, were 10 to 13% of the original total.

Statistical analysis. The data are expressed as the mean plus or minus standard error. The Student t test was used to compare differences between the means.

RESULTS

Effect of PPD on contact sensitivity. Groups of mice were injected i.v. with various doses of PPD 24 h before sensitization. Only in mice given 500 µg of PPD was contact sensitivity significantly depressed compared with the control group, and this dose was therefore used in all subsequent experiments (Fig. 1). The effect of varying the time interval between PPD injection and sensitization was also investigated. Only mice given PPD 24, 48, or 72 h before sensitization exhibited a significant depression of contact sensitivity compared with controls (data not shown). The maximal depression was found when the interval between PPD administration and sensitization was 24 h; this time interval was used in

FIG. 1. Effect of various doses of PPD on contact sensitivity. In all groups PPD was injected 24 h before sensitization, and challenge was performed 6 days later. The shaded area refers to controls consisting of mice that had only been sensitized. The ear swelling of nonsensitized-challenged mice was 2.1 ± 0.6 (10^-3 cm).
PPD-INDUCED SUPPRESSION OF CONTACT SENSITIVITY

FIG. 2. Transfer of depression of contact sensitivity. LN cells (30 x 10^6) from donor mice were transferred into syngeneic recipients 3 days after sensitization. Recipient mice were sensitized 1 h before cell transfer and were challenged 6 days later. OX, Oxazolone; Pcl, picryl chloride.

all subsequent experiments. The effect on contact sensitivity of bovine serum albumin was also investigated. Mice were injected with 500 μg of bovine serum albumin 24 h before sensitization with oxazolone. These animals, challenged with oxazolone 6 days after sensitization, exhibited a response comparable to that of controls (data not shown).

Transfer of suppression. To test whether the suppression of contact sensitivity in PPD-injected mice was mediated by suppressor cells, transfer experiments were carried out. Donor mice, injected with PPD 24 h before sensitization with oxazolone, were killed 3 days after sensitization, and cells from the draining LN were transferred into syngeneic recipients which had been sensitized 1 h before cell transfer. The choice of this time interval between sensitization and cell transfer and of this source of cells was made in view of the fact that in mice the passive transfer of contact sensitivity by

FIG. 3. Characterization of suppressor cells induced by PPD. LN cells (30 x 10^6) from PPD-injected and ox-sensitized mice were transferred into syngeneic recipients 3 days after sensitization. Recipients were sensitized 1 h before cell transfer and were challenged 6 days later.
draining LN cells peaks at day 3 after sensitization (2). LN cells taken from mice that had only been sensitized or given PPD were also transferred into syngeneic recipients. The control group consisted of oxazolone-sensitized mice receiving no cells. Experimental and control groups were challenged 6 days after cell transfer, and the increase in their ear swelling was assessed 24 h after challenge. The results show that only cells from mice that had been both PPD injected and oxazolone sensitized were capable of inhibiting contact sensitivity in the recipients. These cells were antigen specific, in that they failed to suppress the response of recipient mice sensitized with a different skin sensitizer, picryl chloride (Fig. 2).

**Nature of the cells involved in suppression.** Various B-cell-enriched cell subpopulations, obtained from the draining LN of mice which had been sensitized with oxazolone 3 days earlier and also injected with PPD 24 h before sensitization, were transferred into syngeneic recipients which had been sensitized with oxazolone 1 h before cell transfer. Only the B-cell-enriched subpopulation was able to suppress contact sensitivity in the recipients, whereas the T-cell-enriched subpopulation was not (Fig. 3). The suppressive activity was removed after treatment of the whole LN cell population with anti-mouse immunoglobulin serum and complement but not after treatment with anti-Thy 1.2 antibodies and complement. To characterize further the B lymphocytes responsible for suppression, the nylon wool-treated LN B cells were separated by panning into four subpopulations: the anti-ox and anti-Id, B-cell-enriched subpopulations and the anti-ox and anti-Id, B-cell-depleted subpopulations. These were separately transferred into syngeneic recipients sensitized 1 h earlier. The anti-ox B cells inhibited contact sensitivity to oxazolone, as did the anti-Id depleted B cells, whereas the anti-Id B cells and the anti-ox depleted B cells were ineffective (Fig. 4). Neither anti-Pcl nor anti-Id, anti-Pcl B cells affected contact sensitivity to oxazolone (data not shown). These results, however, did not rule out the possibility that the anti-Id B lymphocytes may play a role in PPD-induced suppression at various times after sensitization. To investigate this point, the anti-Id B cells as well as the anti-ox B cells were transferred into syngeneic recipients which had been sensitized 3 days earlier. At this time of the sensitization process, the results observed were exactly the opposite: the anti-Id B lymphocytes were able to transfer suppression, whereas the anti-ox B lymphocytes were not (Fig. 5). Neither anti-Pcl nor anti-Id, anti-Pcl B cells affected contact sensitivity to oxazolone (data not shown).

**Characterization of suppressor cells that inhibit the effector phase of contact sensitivity.** Further experiments were performed to investigate the cells involved in PPD-induced suppression later than day 3 of the sensitization process, i.e., the suppressor cells that inhibit the effector phase of contact sensitivity at day 6 when the challenge was performed. Passive transfer experiments were chosen to mimic the effector phase of contact sensitivity, and \(5 \times 10^7\) LN cells from mice that had been sensitized 3 days earlier were found to be capable of transferring contact sensitivity to normal recipients. Using this model, we investigated the ability of B-

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**FIG. 4.** Characterization of suppressor B cells induced by PPD. Enriched anti-ox and anti-Id B lymphocytes (20 \(\times\) \(10^6\) cells each) from immunodepressed mice were transferred into syngeneic recipients 3 days after their sensitization. Recipients were sensitized 1 h before cell transfer and were challenged 6 days later.
and T-cell-enriched subpopulations taken from immunodepressed mice at day 6 after their sensitization to inhibit the passive transfer of contact sensitivity. Only the T-cell-enriched subpopulation was able to affect passive transfer of contact sensitivity, whereas the B-cell-enriched subpopulation was not (Fig. 6).

**DISCUSSION**

These results show that PPD inhibits contact sensitivity to oxazolone in mice. Transfer experiments demonstrated that the suppression is mediated by anti-ox and anti-Id B lymphocytes as well as by T lymphocytes. These cells, however, exert their suppressive activity at different times during the sensitization process. The anti-ox B lymphocytes appear to play a major role in the early phases of the process, in that they failed to transfer the suppression to syngeneic mice at day 3 after sensitization. Since PPD is a PBA (26), one could postulate that PPD polyclonally triggers B-cell clones to proliferation and that oxazolone selects and expands further the specific B-cell clones, leading to a large number of anti-
oxazolone or through the production of antibodies, may inhibit contact sensitivity by preventing the antigen from reaching the specific reactive T cells. This type of immunoregulation has been proposed for antibodies by several authors (9, 30, 34) and would explain why the anti-ox B cells failed to affect contact sensitivity in syngeneic recipients receiving these cells at day 3 of their sensitization process, i.e., when the effector T cells are already present (22). However, the overall results of the present investigation cannot be explained in light of this regulatory mechanism, in that at day 3 after sensitization, suppression appears to be mediated by anti-Id B lymphocytes. The regulatory role of idiotypic-anti-idiotypic interactions in the contact sensitivity model has already been reported by several authors (22, 28).

In these studies, the anti-Id antibodies were found in the serum of mice 9 to 12 days after sensitization. On the contrary, in our model the anti-Id B-cell subpopulation could already be detected at day 3 after sensitization. Since it has been shown that anti-Id clones are expressed after a polyclonal activation of B lymphocytes by lipopolysaccharide (25), it seems likely that the early appearance of anti-Id B-cell clones may be due to the PPD-induced polyclonal activation, together with the subsequent selection and expansion brought about by the antigen-specific Id+ cells. These latter cells are probably anti-ox, Id+ B lymphocytes, although a contribution from Id- T cells cannot be excluded (22). Moreover, the anti-ox Abs produced by the expanded specific B-cell clones may lead to the formation of immune complexes, which are known to represent a further stimulus for the production of anti-Id antibodies (17). However, it remains to be clarified why anti-Id B lymphocytes failed to inhibit contact sensitivity at the moment of sensitization and exerted their suppressive effects at day 3 after sensitization. The ineffectiveness of anti-Id B lymphocytes at the time of sensitization might be related to a decay of these cells occurring in the absence of an adequate stimulus, i.e., Id+ lymphocytes, which are unlikely to be formed to a sufficient extent until 2 to 3 days after sensitization. The ability of anti-Id B lymphocytes to affect contact sensitivity at day 3 after sensitization can be explained if one considers the mechanism of action of these cells. In some systems, anti-Id antibodies act by blocking antigen receptors on effector T cells (18), whereas in others they act indirectly by triggering suppressor T cells (27, 32). The results of the present investigation are in favor of the second of these possibilities, since B lymphocytes were unable to block the T lymphocytes mediating the passive transfer of contact sensitivity. An important question which cannot be answered by the present results is whether PPD inhibits contact sensitivity through the amplification of immunoregulatory circuits that arise under conventional immunization (1, 33) or through the activation of regulatory cells which are not usually seen after exposure to contact sensitizer. Experiments are currently underway to investigate this point.

In conclusion, the data suggest the following. PPD activates B cells polyclonally; oxazolone, given 24 h later, selects and further expands the specific B-cell clones which, in turn, select and expand the corresponding anti-Id B-cell clones already activated polyclonally by PPD. The anti-Id B-cell subpopulation, through the production of anti-Id antibodies, finally activates suppressor T cells that inhibit the effenter phase of contact sensitivity. Although further studies are in progress to obtain conclusive proof of this hypothesis, the results of the present investigation clearly indicate that a PBA induces an early activation of the immunoregulatory circuits of the host. An early interference of PPD with these circuits may have a great importance in mycobacterial infections. The induction of suppressor cells that inhibit cell-mediated immune responses could account for anergy in patients with active tuberculosis (5, 16) and may represent a way of depressing the specific defenses of the host by mycobacteria.

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