SITE OF ACTION OF SERUM FACTORS THAT BLOCK
DELAYED-TYPE HYPERSENSITIVITY IN MICE*

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Delayed-type hypersensitivity (DTH)1 develops without adjuvant if the dose
of SRBC given is appropriate to the route of inoculation (1). Increasing the
amount of antigen reduces and eventually abolishes all evidence of DTH.
Animals blocked by an excessive dose of SRBC cannot be subsequently sensi-
tized, even when an optimal dose of antigen is given subcutaneously, the most
reliable route for inducing DTH in the mouse. DTH does develop, however,
when a massive intravenous dose of antigen is given to splenectomized mice, or
mice that have received cyclophosphamide before immunization (2). The sup-
pression of DTH cannot therefore be due to excess antigen, as such. The serum
of DTH-suppressed mice will partially inhibit the induction and expression of
DTH in normal recipients, an effect which is substantially increased by partial
absorption of the serum with specific antigen (3). In contrast to a high dose of
antigen (4), absorbed serum does not inhibit the proliferative response or the
formation of plaque-forming cells (PFC) in lymph nodes draining the site of
antigen injection. Moreover, the specific antibody titer is increased by absorbed
blocking serum even though the induction of DTH is totally suppressed by it.

In light of indications that the products of the interaction between antigen
and antibody depress the activated T cells which mediate DTH, and insofar as
pretreatment with specific IGM antibody has been reported to enhance the
antibody response (5, 6), an important question arises concerning the functional
relationship between the mediators of DTH and the T cells responsible for
helper activity in the antibody response. Obviously, a successful antibody
response depends upon cooperation between the various cellular and humoral
elements involved (7). It is possible that this can be accomplished efficiently
only in certain sites, and that the local enactment of a DTH reaction is the
process whereby the participating elements are assembled (8). After intrave-
nous immunization, the spleen could be the locus of this coordinated series of
events. Evidence in support of this concept is presented in the present report,

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1 Abbreviations used in this paper: AS4, S4 after absorption with an equal volume of packed
SRBC; CRBC, chicken erythrocytes; CY, cyclophosphamide; DTH, delayed-type hypersensitivity;
PFC, plaque-forming cells; S4, serum obtained 4 days after intravenous immunization of mice
with 109 SRBC; SRBC, sheep erythrocytes.
which deals with the site of blocking by immune serum of the cells that mediate DTH to sheep erythrocytes (SRBC) in mice.

**Materials and Methods**

**Animals.** Except when cell transfers were made, the mice used were specific-pathogen-free mice of the outbred CD-1 strain (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). Adoptive sensitization by spleen cell or lymph node cell transfer was performed syngeneically, using strains B6H-T~ F1 (C57Bl/6 × CBA H-T6) or C57Bl/6 mice from the breeding facilities of the Trudeau and Pasteur Institutes. Mice were used at 5 or 8 wk of age.

**Antigens.** SRBC were obtained from the same animal once weekly. Cells were collected and stored in Alsever's solution at 4°C. Chicken erythrocytes (CRBC) were obtained and stored in the same way. Cells were washed three times with normal saline and suspended to known density by hemocytometer count.

**Cyclophosphamide (CY).** CY was donated by Mead Johnson & Co. (Evansville, Ind.) and by Lab. Lucien (Colombes, France). A single dose (200 mg/kg) was given intravenously before immunization (2), though intraperitoneal or subcutaneous routes gave identical results.

**Immunization.** Except when specified, 10⁸ SRBC were used for primary immunization by intravenous or by foot-pad inoculation. For modulation of the primary response, cyclophosphamide was administered 2 days before intravenous immunization or concurrently with subcutaneous immunization.

**Test for DTH.** DTH was measured after 24 h as the increase in foot-pad thickness elicited by an injection of 10⁶ SRBC in a 40-μl volume (1).

**Splenectomy.** Splenectomized mice were prepared 3 wk before an experiment by removing the spleen through a flank incision. The spleen was dissected from its pedicle and excised without attempting to prevent bleeding. The wound was closed by Mischel clips.

**Blocking Serum.** Serum containing specific inhibitors of activated T cells was prepared from mice bled 4 days after intravenous immunization with 10⁶ SRBC. Its blocking activity was increased by partial absorption with packed SRBC as previously described (3). Hereafter, 4-day serum and absorbed 4-day serum are referred to as S4 and AS4, respectively.

**Immune Cell Transfer.** Spleen cells for transfer to syngeneic recipients were prepared as in a previous study (9). The cells were pooled, washed twice, and enumerated in a hemocytometer. Viability, as assessed by dye exclusion with trypan blue, was not less than 90%. For the systemic cell transfer, mice received 0.5 ml of spleen cell suspension intravenously. Recipients were tested immediately for DTH by foot-pad injection of 10⁶ SRBC. For the local transfer of DTH, the method of Metaxas and Metaxas-Bühler (10) was modified slightly; the specifically sensitized lymphocytes and antigen were mixed and then introduced into the foot-pad of the normal or treated recipients.

**Results**

**Adoptive Transfer of DTH by Spleen Cells from Anergic Mice.** Hypersensitivity to SRBC can be transferred adoptively with isolated spleen cells from immunized donors. In the mouse, the active cells bear the alloantigen Thy-1 (11). Those mediating specific sensitivity to SRBC perform well in normal recipients, where they survive with an apparent half-life of about 45 days (12). It has been observed, however, that reactive cells confer no DTH upon recipients which have received 10⁶ SRBC 4 days before cell transfer (3). Similarly, DTH did not appear, and a regional antibody response did not develop in mice given an optimal foot-pad dose of SRBC within 3 days of receiving a large (10⁶) intravenous dose of SRBC (1). On the other hand, serum of such mice, partially absorbed with SRBC, blocked the induction and expression of DTH (3), but did not inhibit the proliferative response or PFC production in the draining node. It is evident, therefore, that the effects of SRBC and AS4 on the production and circulation of activated T cells are significantly different. As a first step towards explaining this difference, a study was made of the capacity of spleen cells from
blocked mice to mediate DTH by local and systemic transfer. It was reasoned that local transfer would test the intrinsic capacity of donor T cells to mediate a DTH reaction, whereas systemic transfer should reveal, in addition, their ability to reach the site of antigen deposition.

Using this approach, the kinetics of the blocking action of an intravenous inoculum of $10^8$ SRBC was studied by adoptive transfer of spleen cells harvested at varying intervals after immunization. The purpose of the experiment was to determine whether reactive cells are produced at all, or whether they are produced and subsequently inhibited. The results (Fig. 1) show that although the cell donors were completely anergic, cell recipients gave reactions which were highly significant after both local and systemic transfer of cells harvested on days 8 and 10 ($P < 0.002$). The local transfer was usually more effective, even though the number of cells transferred was less. The difference between the DTH reactions elicited in donors and recipients of locally transferred cells was statistically significant ($P < 0.05$) at all times through day 14. It is concluded, therefore, that the mediators of DTH are present in the spleens of suppressed mice. It is apparent that they are either inhibited in situ or are prevented from leaving the spleen. In either case, the evidence of a previous experiment suggests that the mechanism involved is cyclophosphamide-sensitive (2). The next experiment was designed to examine the first of these possibilities by testing the capacity of blocking serum to interfere with specifically sensitized lymphocytes at the site of a local cell transfer.

Absence of a Direct Effect of Blocking Serum on the Mediators of DTH. Since it has been reported that immune complexes can bind to activated T cells (13), and that some T cells have receptors for IgM (14–16), an experiment was...
designed to test whether AS4 is adsorbed to activated T cells, thus rendering them functionally inactive, as suggested by Sinclair et al. (17).

Spleen cell suspensions from intravenously immunized mice were washed twice in medium 199. The pellets of pooled spleen cells were resuspended in AS4 or in normal serum in the proportion of 1 ml of serum per spleen equivalent. The suspensions were incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO₂. The spleen cell preparations were then washed three times in medium containing 1% fetal calf serum at 4°C. The supernates after the first wash were kept and subsequently tested for blocking activity. The serum-treated cells were injected intravenously into normal recipients which were tested immediately for DTH by foot-pad inoculation. The supernates (post-absorption serum) were transferred into normal mice that had also received an aliquot of immune cells which had been treated with absorbed normal serum.

Fig. 2 shows, as expected, that the blocking factors in AS4 inhibited DTH to SRBC (P < 0.001), and that blocking was specific since immune serum absorbed with CRBC did not block the reaction in SRBC-sensitized mice (P = 0.4). Although not recorded in Fig. 2, the inverse was also true: AS4 (CRBC) blocked DTH to CRBC but not to SRBC. However, spleen cells incubated with homologous blocking serum showed no change in viability and the specifically reactive component remained intact, as shown by their undiminished capacity to confer DTH upon normal recipients. Moreover, the supernate (post-absorption serum) had not lost its T-cell inhibition as a result of absorption (P < 0.005) (Fig. 2). These results suggest that specifically sensitized lymphocytes are not inhibited, at least in vitro, by direct interaction with specific blocking factors in AS4.

Indirect Effect of AS4 on the Mediators of DTH. The preceding experiment did not exclude the possibility that union between AS4 and activated T cells is unstable, and that treated cells could regain functional activity after transfer. This would be less likely to happen if AS4 were mixed with the specifically sensitized cells and introduced directly into the test-site, as in the Metaxas and Metaxas-Bühler technique for transferring DTH (10). This possibility was examined in groups of mice that were adoptively immunized by either systemic or local cell transfer.

As described in the legend of Fig. 3, AS4 blocked DTH only when given intravenously to recipients immunized by systemic cell transfer (P < 0.001); it failed, almost completely, to prevent DTH when it was introduced locally with the sensitizing cells. Conversely, AS4 given intravenously did not block the local transfer of DTH, but did affect systemic transfer to some extent when given subcutaneously. This seems to say that there is a geographic restriction to the blocking action of AS4. It can be concluded, in fact, that AS4 actively blocks DTH only when AS4 is present in the systemic circulation with the specific mediators of DTH.

It should be noted, too, that AS4 given intravenously did not interfere with the local transfer of DTH. This means that blocking serum does not affect the migratory behaviour of the accessory cells (monocytes) which are necessary participants in a DTH reaction. This appears to implicate the T cell as the victim of the blocking reaction.

In an additional experiment, SRBC and immune serum (S4) were mixed as for the production of blocking serum (AS4), and injected into the foot-pads of
hypersensitive mice. The mixture elicited reactions greater than those produced with antigen alone, probably because the SRBC were more effectively retained at the inoculation site. The experiment showed again that mediator cells are not directly inhibited by factors present in blocking serum. It was interesting to find, however, that AS4 failed to elicit a DTH reaction even though it provoked a PFC response in the regional node (P. H. Lagrange, unpublished observations). This suggests that free antigen is needed for eliciting a DTH reaction but not for the induction of an antibody response, at least in hypersensitive mice.

_Localization of Site of Action of AS4._ We have shown that DTH develops in splenectomized mice even when blocking doses of antigen are administered (1). The same conditions prevail in animals given a dose of CY sufficient to prevent antibody formation. Being the major organ of both antibody formation (18) and the regulation of some T-dependent immune responses (19), the spleen thus seemed a likely anatomical site for the event that results in blocking of DTH. This possibility was tested in a composite experiment in which blocking by AS4
Fig. 3. In an experiment of design similar to that of Fig. 2, the blocking of DTH was shown to be geographically restricted to cells in the systemic circulation. The sensitized donors (D) and a group of unimmunized controls (C) were tested for DTH on day 5. The remaining donors were used to prepare suspensions of normal or sensitized spleen cells for systemic or local transfer to normal recipients. The systemic recipients received one spleen equivalent of normal (C) or sensitized cells intravenously. Before testing for DTH, mice of separate groups were given 0.2 ml of normal serum (NS) or AS4. The former was given intravenously, and the latter intravenously (iv) or subcutaneously (sc) in the neck. Even when administered subcutaneously, AS4 depressed the foot-pad reaction in systemically sensitized mice. With local transfer of sensitized cells, the effect was different. Whether given intravenously or into the reaction site with spleen cells and antigen, AS4 caused no significant depression of DTH. Groups of 5 ± SEM.

was compared in normal, splenectomized, and CY-treated recipients of SRBC-sensitized spleen cells.

The results of the experiment described in Fig. 4 show the reactivity to SRBC in the donor mice and in recipients given varying numbers of immune spleen cells intravenously. It can be seen that undiminished reactions were elicited in splenectomized or CY-treated recipients of $10^8$ immune spleen cells that had been treated with normal mouse serum. However, in groups of similar recipients that had received AS4 instead of normal mouse serum, significant blocking of DTH occurred only in normal recipients. The effect of AS4, which was almost abolished by splenectomy or CY alone, was completely abolished in animals that were both splenectomized and treated with CY. It will be noted from Fig. 4 that the dose of AS4 administered was enough to reduce the apparent level of T-cell activity in normal recipients by a factor of $10^3$. Thus, it seems that blocking factors in AS4 interfere with the function of the mediators of DTH through an event occurring in the spleen and involving CY-sensitive cells. Trapping of activated T cells in the spleen suggests itself as a likely mechanism.

Discussion

The present experiments begin by demonstrating that the absence of DTH
after immunization with an excessive dose of antigen cannot be due to the absence of sensitized cells, because cell transfer showed that reactive cells were present in the spleens of anergic mice. It was then shown in several ways that the blocking factors which appear in serum do not interfere directly with the function of mediator cells. In fact, blocking could be demonstrated only when blocking factors and reactive T cells were present together in the systemic circulation. It must be emphasized, however, that this statement applies only to T cells in a recently activated state. In effect, this means mitotically active cells, because it was demonstrated previously that only vinblastine-sensitive cells are susceptible to blocking by AS4 (13).

The finding that blocking factors are specific for homologously reactive T cells, and can function only in the presence of an intact spleen, implies that suppression of DTH is due to an immunologically specific event involving the spleen. The most likely role for the spleen in blocking DTH is that of selective trapping of mediator cells. Experiments to demonstrate specific trapping of T cells have been reported (17, 20, 21), but they have relevance to the inductive phase of an immune response rather than to the mediators of DTH. The present experiments demonstrate quite clearly that the spleen has a part to play in the suppression of DTH which is observed after administration of large quantities of antigen. They also show that the underlying mechanism is ablated by prior treatment with cyclophosphamide. This implies the participation of a drug-sensitive population of cells. Four cell types deserve consideration.

B-Cell Precursors. It has been shown by several investigators that cyclo-
phosphamide depresses antibody production preferentially when given before antigenic stimulation (2, 22-26). It is likely, therefore, that after cyclophosphamide the capacity to produce antibodies would have been depressed in animals that had been adoptively sensitized with SRBC-specific T cells and given blocking serum intravenously. Since blocking serum contains specific antibody, it is obvious that blocking is not due to antibody per se. Hence, by inference, the absence of blocking in CY-treated animals cannot be simply due to the prevention of antibody production by the transferred cells. This makes it difficult to directly implicate B cells in the blocking process.

**Monocyte Precursors.** The monocyte precursor in bone marrow is radiosensitive (27) and susceptible to cyclophosphamide (2, 28, 29). It is well known that monocytes are essential to the enactment of a DTH reaction in the skin (30) or foot-pad (27). It is conceivable, therefore, that the uptake of antigen by the spleen after intravenous immunization would serve to elicit the equivalent of a DTH reaction in that organ, with a resulting retention of specifically reactive lymphocytes and an accompanying accumulation of monocytes. Such an event would obviously limit the availability of cells in circulation for a reaction in the foot-pad, for example. It was observed, however, that a local transfer of DTH was possible in systemically blocked mice (Fig. 3). Obviously, retention of monocytes by the spleen cannot explain the blocking phenomenon.

**Suppressor Cells.** There is substantial evidence for the existence of suppressor T cells. They are usually detected through their capacity to interfere with antibody production (31). It has been shown more recently, however, that T cells with a capacity to suppress cell-mediated hypersensitivity also appear in the spleen under the conditions that lead to suppression of DTH (32). Therefore, it must be admitted that a suppressor cell, which is sensitive to cyclophosphamide, could be involved in the blocking of DTH (33). If so, it must be assumed that blocking factors actually induce the formation of the suppressor cells and that the inductive process is very rapid because blocking is complete and instantaneous in adoptively sensitized animals. Moreover, it can be deduced from the present experiments that a suppressor cell, if it exists, is not available in the circulation to exert its suppressive effect on a DTH reaction elicited in the foot-pad. The results of experiments designed to evaluate the role of suppressor cells in the blocking of DTH will be reported in a subsequent paper.

**Dendritic Cells.** The dendritic cell, which has a known capacity to trap antigen-antibody complexes at the cell surface (34), is very sensitive to radiation (35). It might therefore be sensitive to a radiomimetic drug such as CY, although no evidence for this has been reported. Since a low intravenous dose of SRBC (10⁶-10⁷) causes T-cell activation and DTH without causing antibodies to be formed (including an absence of PFC), whereas larger doses cause antibody production without DTH even though the activated T cells are present in the spleen (Fig. 1), it seems plausible that the association between activated T cells and antibody-forming B cells may be more than circumstantial: it may represent a true physical relationship which could involve the dendritic macrophage as a focus for the cellular cooperation that results in antibody production. It may be, in fact, that the germinal center is a morphological structure created to satisfy the necessity for cooperation between cell types. According to this hypothesis, blocking factors, which are believed to be a complex of antigen and IgM, would
become associated with dendritic macrophages, thus creating the attractive focus that prevents the release of activated T cells from the spleens of massively immunized mice, or causes them to become trapped in that organ after the passive administration of AS4 to adoptively sensitized subjects. If such a mechanism exists, the molecular mechanism and the relevant receptors involved in cell-cell binding have yet to be elucidated.

There is a long history to the close functional relationship that seems to exist between the phenomenon of DTH and the mechanism of antibody formation (8, 36). It has been suggested, in fact, that when a DTH reaction is observed in the spleen it is a displaced expression of a physiological process that normally occurs in lymphoid tissue during the inductive phase of an antibody response. Viewed in this way, the blocking of DTH in animals immunized with a large intravenous dose of antigen cannot be thought of as suppression of a particular form of immunological reactivity, but rather as the redirection of cells (37) from one possible role as the mediators of DTH to an alternative role as helper cells in antibody formation. This, however, may be far too simplistic an explanation for the observed effects of serum factors on the expression of DTH in mice. It is consistent, however, with the frequent coexistence of specific anergy and hypergammaglobulinemia.

Summary

When mice have been rendered anergic by a large intravenous dose of sheep erythrocytes, their inability to mount a delayed-type hypersensitivity (DTH) reaction is not due to an absence of mediator cells, for these can be detected in the spleen by cell transfer. Nor is it due to disappearance of accessory cells (monocytes) from circulation. The serum of anergic mice contains blocking factors which are more abundant after absorption with antigen. Such factors are unable to inactivate the mediators of DTH in vitro, nor do they suppress a DTH reaction when introduced locally into the reaction site. They are active, however, when given intravenously to systemically sensitized mice, provided that the sensitized animal has an intact spleen. If the spleen has been removed or the recipients of sensitized cells have been treated with cyclophosphamide before cell transfer, blocking factors are no longer able to suppress a DTH reaction. Reasons are given for the belief that suppression of DTH in animals undergoing a vigorous antibody response is due to the diversion of reactive cells from circulation to undertake an alternative role in antibody formation in the spleen.

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