Delayed-type hypersensitivity (DTH) appears in mice immunized with sheep red blood cells (SRBC) if the dose of antigen is matched to the route of immunization (1). The optimum intravenous dose for sensitizing CD-1 mice is $10^8$ SRBC. As the dose is raised above this optimum, DTH diminishes, disappearing completely beyond $10^8$. No subsequent dose of SRBC, by any route, will induce DTH once T cells have been blocked by previous immunization. Blocking is due to a product of the interaction between antigen and antibody (presumably an immune complex) (2). The formation of specific antibody can be temporarily prevented by cyclophosphamide (CY) (3), thus liberating the T-cell response from feedback inhibition and allowing DTH to reach abnormal levels in response to doses of antigen that would abolish DTH completely in untreated mice (4).

A systemic infection with BCG also amplifies the T-cell response to SRBC. It does so by interfering with the blocking mechanism that normally limits the T-cell response (5). The effects of CY and BCG, being additive, combine to produce spectacular levels of DTH which may persist for 6 mos or more. Augmentation of the T-cell response to a first encounter with antigen may be less important, however, than the problem of reestablishing cell-mediated immunity after T cells have become blocked by a humoral response. We need measures, for example, that will rescue T cells from subjugation by products of the humoral response in tumor-bearing (6) or chronically infected (7) subjects.

**Materials and Methods**

All materials and methods have been described (1, 2, 4, 5, 8, 9).

**Animals.**—Specific pathogen-free mice of the CD-1 strain (Charles River Breeding Laboratories, Wilmington, Mass.) were used at 5–6 wk of age.

**Mycobacterium Bovis BCG.**—The Pasteur strain of BCG (Trudeau Mycobacterial Collection no. 1011) was cultivated in Middlebrook's 7H9 medium (Difco Laboratories, Detroit, Mich.) and frozen while in log-phase growth. It was preserved at $-70^\circ$C in vials containing $4 \times 10^8$ viable BCG/ml.

**Cyclophosphamide.**—Cytoxan (CY) was donated by Mead Johnson and Co., Evansville, Ind. It was dissolved in sterile saline (20 mg/ml) and administered intravenously as a single dose (200 mg/kg).

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† Lillia Babbitt Hyde Fellow for 1974.

‡ Lagrange, P. H., and G. B. Mackaness. 1974. A stable form of delayed-type hypersensitivity. Manuscript in preparation.
**Delayed-Type Hypersensitivity.**—DTH was measured as the increase in footpad thickness 24 h after inoculation of an eliciting dose of $10^7$ SRBC (9).

**RESULTS**

DTH was blocked on day 0 by a massive intravenous injection of $10^7$ viable BCG Pasteur intravenously on day 14. Half of these and an equal number of uninfected mice were given CY on day 30. On day 30, mice of all groups (untreated, BCG, CY, BCG + CY) were subdivided and injected intravenously with a second antigenic stimulus of varying intensity ($10^4$–$10^6$ SRBC). DTH was measured 6 days later in the right hind footpad.

Fig. 1a shows that previously immunized mice did not develop DTH in response to any of the doses of antigen used for the secondary stimulus. But mice given either CY or BCG developed some degree of DTH. Those given both BCG and CY, however, responded vigorously; behaving, in fact, as if they had never previously encountered SRBC (5). The hemagglutinating titer at the time of attempted rescue of T-cell activity was 1:1024.

A second attempt to restore T-cell activity was begun 60 days after the primary (suppressing) dose of $10^7$ SRBC. The antibody titer was still 1:512. Fig. 1b shows that the response to reimmunization with $10^8$ SRBC produced

![Graphs showing DTH responses](image-url)

**Fig. 1.** (a) Animals which have previously been immunized with a T-cell-blocking dose of SRBC ($10^7$) will not subsequently respond with the development of DTH to any dose of SRBC in the range $10^5$–$10^6$ (*—*). Treatment with CY (▲—▲) 2 days before, or infection with BCG (●—●) 12 days before antigenic stimulation, allowed some degree of T-cell activity to emerge. Treatment with both agents (■—■), however, restored the response almost to normal (5). Means of five ± SEM. (b) The rate of development, magnitude, and duration of the DTH provoked by the highest ($10^8$) dose of SRBC in mice belonging to the four groups of mice described in (a) above. Means of five ± SEM.
peak levels of DTH that were not much less than those achieved by animals responding for the first time under identical conditions of treatment with BCG and CY to the same dose of SRBC (5).

The Suppression of T-Cell Activity During Prolonged Exposure to Antigen.—Beginning with an initial dose of 50 SRBC, mice were exposed to gradually increasing amounts of antigen by successive twofold increments in the daily dose of SRBC. The first 10 injections were given intravenously, the rest intraperitoneally. At 5-day intervals, groups of five mice were tested for DTH, and then were bled to determine the prevailing levels of hemagglutinating antibody.

Fig. 2a shows that the high point of T-cell reactivity was achieved on day 10, when the cumulative dose of SRBC had reached about $10^5$. Antibodies were still not present at this time, but the level of DTH was much less than would have been reached within 4 days of injecting a single pulse of $10^5$ SRBC (1). Antibody appeared in the serum after day 10 and DTH began to decline.

![Graph of DTH measurements](image)

**Fig. 2.** (a) DTH was measured at 5-day intervals (●—●) in groups of mice drawn from a much larger pool of animals which received SRBC in twofold increments on each successive day. The cumulative dose of SRBC is represented by the interrupted line. The total and mercaptoethanol-resistant hemagglutinin titers (log2) at each time point are recorded beside the mean (±SEM) footpad reactions elicited in groups of five mice. (b) Mice which had become anergic after receiving a total dose of $10^9$ SRBC were used to test the capacity of BCG and CY to restore T-cell responsiveness. The experimental design is described in the text. Tests for DTH were performed 6 days after restimulation with $10^8$ SRBC. Mice not given this resuscitating intravenous injection of antigen are represented by the black columns. Means of five ± SEM. The total/2 ME-resistant antibody titers for the pooled serum from each group of mice (above each column) show that the secondary antibody response was suppressed in all CY-treated mice.
until it had disappeared completely by day 25 when the cumulative dose of SRBC had reached $10^9$.

Restoration of T-Cell Activity to Anergic Mice.—As might happen during growth of a tumor or in the course of an infection, the mice exposed to a mounting concentration of antigen passed through a hypersensitive phase before becoming completely anergic. The untested mice remaining in the experiment were therefore good subjects for an attempted rescue of T-cell activity.

After DTH had disappeared on day 25, antigen injections were stopped and the mice were rested for 5 days before commencing the rescue operation. On day 30 some of the suppressed mice were injected intravenously with $10^7$ BCG; and after another 20 days half of these, and an equal number of uninfected mice, were treated with CY. 2 days later, a secondary immunizing stimulus of $10^8$ SRBC was given intravenously to all mice except for those in two additional groups: one untreated and the other given both BCG and CY. These were deliberately not given the resuscitating antigenic stimulus in order to determine whether DTH would return spontaneously, with or without the help of BCG and CY.

Fig. 2 b shows that mice which were made anergic by a continuously increasing exposure to SRBC were still anergic when tested 25 days after antigen injections had ceased. Moreover, they did not develop DTH in response to a resuscitating dose of antigen (Fig. 2 b, untreated). Mice given CY 2 days, or BCG 20 days, before reimmunization developed some degree of DTH; while those treated with both BCG and CY became intensely hypersensitive. It will be noted, however, that DTH did not appear spontaneously. It required further specific antigenic challenge.

DISCUSSION

The present experiments have shown that cell-mediated immunity can be reestablished in animals which have already responded with antibody production and are apparently refractory to the further induction of DTH. Refractoriness could be lifted partially by cyclophosphamide or BCG; but together these agents freed the T-cell response completely (Fig. 1). It was even possible to restore T-cell activity to animals which had been kept under continuous antigenic stimulation for almost 4 wk (Fig. 2).

The artificial conditions provided by these experimental models resemble two important clinical situations. The repeated injection of gradually increasing numbers of SRBC serves to simulate conditions in tumor-bearing animals and in some infections in which exposure to antigen begins insidiously but becomes intense. The findings with this model (Fig. 2) make two potentially significant points: that the ultimate level of DTH reached was low in animals exposed to a gradually increasing amount of antigen; and that DTH does not return spontaneously, even under the influence of BCG and CY. For some reason, the slowly mounting stimulus did not permit full expression
of T-cell activity before the inhibitory mechanism that blocks cell-mediated immunity (2) began to assert itself. Had mice been injected with $10^5$ SRBC intravenously or $10^7$ SRBC subcutaneously, they would have achieved much higher levels of DTH (5). This shows that the cell-mediated attack on a source of antigen, whether it be a tumor or a slowly enlarging population of microorganisms (as in leprosy), may be interrupted prematurely if the antigenic stimulus develops too slowly. This has obvious significance in relation to the concept of "sneak-through" in tumor immunology (10).

The same experiment showed that T-cell activity did not return spontaneously, even to BCG- and CY-treated animals. To restore T-cell activity, a new antigenic stimulus had to be applied, but the response obtained was essentially undiminished compared with that of previously unimmunized animals (5). Clearly the refractory animal suffers no defect in immunocompetence, it is merely subject to a very efficient regulatory mechanism that precludes the induction of cell-mediated immunity.

The presumed basis for these restorative effects of BCG and CY are the capacity of cyclophosphamide to interfere with feedback inhibition of T cells by its influence on antibody formation (4); and the ability of BCG, acting perhaps to clear the circulation of immune complexes, to permit the immune response a longer period of evolution in the relative absence of feedback inhibition (5). The results show clearly that massive amounts of antigen do not rob an animal of competent T cells but merely prevent the emergence of cell-mediated immunity by premature inhibition. BCG, however, has other possible modes of action (5, 9), including one that postulates the production of an "unblocking" antibody (11).

Whatever the mechanism, the present observations show that it is possible to abolish the anergic state in an artificially suppressed animal, and encourages the thought that it may be possible to achieve similar effects in comparable clinical situations. It is apparent, however, that heroic measures, such as an intravenous injection of living BCG and a near lethal dose of CY, may be needed.

SUMMARY

The T cells which mediate delayed-type hypersensitivity (DTH) to sheep red blood cells (SRBC) are blocked by a normal humoral response and cannot be made to function by further immunization. They can be rescued to some extent by treatment with immunopotentiating agents such as cyclophosphamide (CY) which suppresses the antibody response selectively, or by BCG which interferes with the action of serum blocking factors. These two agents together can restore cell-mediated immunity completely, but a further antigenic stimulus is needed to reestablish DTH in mice blocked by a long period of continuous exposure to SRBC.
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