HETEROGENEITY OF THE CELLULAR IMMUNE RESPONSE

I. KINETICS OF LYMPHOCYTE STIMULATION DURING SENSITIZATION AND RECOVERY FROM TOLERANCE*

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(Received for publication 9 September 1970)

Immunization with a foreign protein stimulates the production of a heterogeneous group of immunoglobulins differing in class, allotype, and avidity for specific antigen. Studies with hapten–protein conjugates have demonstrated a wide range of hapten-binding energies among subfractions of a single antiserum (1, 2). After immunization, the average affinity for hapten increases with time and the rate of increase can be maximized with an optimal immunizing dose (3). Conversely, the antibody produced by partially tolerant animals is of low average affinity (4–6).

Siskind and Benacerraf (7) have proposed that the increase of antibody affinity observed after immunization reflects the selective pressure of antigen on a heterogeneous population of immunocytes, each bearing a surface receptor resembling the antibody it will synthesize. According to their model, cells with receptors of high affinity proliferate at low antigen concentration, whereas cells with low affinity receptors require high levels of antigen for comparable stimulation. As the concentration of available antigen falls progressively after sensitization, proliferation of high affinity cells would be favored, accounting for the observed increase in average affinity of circulating antibody. Conversely, if tolerance is induced with a large dose of antigen, cells of high affinity, capable of binding antigen most avidly, would be selectively suppressed.

Evidence for heterogeneity among antigen-sensitive cells has been drawn from observations in vivo of cross-reactivity between closely related hapten–protein conjugates and from the dose-response characteristics of cell-mediated reactions (8–16). In vitro studies by Paul et al. (10) have demonstrated a gradual and progressive increase of thymidine incorporation by sensitized lymphocytes on exposure to specific antigen over an extremely wide range of concentration. Immunization with high doses of antigen, known to evoke antibody of low average affinity, selected a population of...
cells requiring high concentrations of antigen for optimal stimulation in culture. Sensitization with smaller amounts of antigen permitted optimal reactivity at lower antigen concentration, associated with antibody of high average affinity. However, these authors did not detect a fall in the antigen concentration necessary to elicit minimal or half-maximal stimulation at late intervals following sensitization, corresponding to the marked increase in antibody affinity observed over the same period.

In this paper we provide additional evidence for heterogeneity in the response of lymph node cells from sensitized and tolerant guinea pigs. An evolution of cellular reactivity was observed after immunization with human serum albumin (HSA) in Freund's complete adjuvant, and this evolution was retarded by the induction of tolerance. Antigen-specific stimulation of thymidine incorporation occurred at progressively lower concentrations of HSA at successive intervals following sensitization, associated with a concomitant increase in the magnitude of maximal stimulation. Lymph node cells from animals rendered tolerant to HSA failed to react with specific antigen in culture. As tolerance waned, reactivity was first observed at high, but not at low antigen concentrations. Finally, the dissociation of delayed hypersensitivity and antibody formation observed early in the immune response and on recovery from tolerance has provided further correlation of lymphocyte stimulation with delayed cutaneous reactivity (17, 18).

Materials and Methods

Antigens.—HSA, crystallized, lots 24-27, was obtained from Pentex, Inc., Kankakee, Ill. Preservative-free purified protein derivative (PPD) was the generous gift of Parke, Davis & Co., Inc., Detroit, Mich. Old tuberculin (OT) was supplied by the Massachusetts Department of Health. Antigens were dissolved in either sterile pyrogen-free saline or in Eagle's minimum essential medium with glutamine (MEM) (Grand Island Biological Co., Grand Island, N.Y.) and were passed through a sterile 0.22 μM Millipore filter that had been washed exhaustively to remove contaminating detergent (19).

Immunization of Guinea Pigs.—450-500 g male Hartley guinea pigs (Camm Research Institute, Inc., Wayne, N.J.) received 10 μg HSA emulsified in 0.1 ml complete Freund's adjuvant (CFA) divided among the four footpads. Control groups received 0.1 ml CFA without HSA. CFA contained 50 parts saline, 42.5 parts Bayol 55 (Esso Chemical Co., Rahway, N.J.), 7.5 parts Arlacel A (Hilltop Laboratories, Inc., St. Paul, Minn.), and tubercle bacilli (human strains C, DT, and PN, Mass. Dept. of Health) to provide a final concentration of 3 mg/ml. In some experiments the concentration of bacilli was reduced 10-fold to 300 μg/ml (CFA 1:10). Tolerance was produced by the intravenous injection of either 500 μg or 5 mg HSA in 1.0 ml saline, immediately before immunization with the same antigen in adjuvant (20).

Skin Tests and Antibody Assay.—Skin tests were performed by the intradermal injection of 0.1 ml of saline containing either 6 μg HSA or 1:100 OT. Reactions were read at 24 hr.

Abbreviations used in this paper: CBH, cutaneous basophil hypersensitivity; CFA, complete Freund's adjuvant; dpm, disintegrations per minute; HSA, human serum albumin; MEM, Eagle's minimal essential medium; NGPS, normal guinea pig serum; OT, old tuberculin; PCA, passive cutaneous anaphylaxis; PPD, preservative-free purified protein derivative.
recording the diameter and intensity of erythema, the extent of induration (0 to 4+), and the presence or absence of necrosis. For histologic examination, skin reactions were excised following sacrifice and 1 μ sections prepared (21).

Sera were assayed by passive hemagglutination which detected both 7S1 and 7S2 antibodies. 7S2 antibodies were assayed directly by passive hemolysis; 7S1 antibodies were measured by passive cutaneous anaphylaxis and in some cases by systemic anaphylaxis following intravenous challenge with 5 mg HSA.

Lymphocyte Culture.—Guinea pigs were anesthetized with ether and exsanguinated. Subscapular, axillary, inguinal, femoral, and popliteal lymph nodes were excised with aseptic technique and placed in MEM containing penicillin and streptomycin. Nodes trimmed of fat were transferred to antibiotic-free MEM supplemented with 10% heat-inactivated normal guinea pig serum (NGPS). Cells were teased into suspension, filtered through sterile 100 mesh wire cloth, and washed three times in MEM with 10% NGPS with centrifugation at 800 rpm for 10 min at ambient temperature. After a final resuspension in MEM with serum, cells were counted and viability assessed with 0.1% trypan blue. An average of 570 X 10^6 lymph node cells were isolated per animal with a mean viability of 63%. No significant difference in total cell yield per animal was found among different experimental groups. Replicate cultures were prepared in 16 X 125 mm plastic culture tubes (Falcon Plastics, Los Angeles, Calif.). Each contained 1 X 10^7 viable nucleated cells in a final volume of 2.5 ml. MEM supplemented with 20% NGPS, 100 units/ml penicillin and 100 μg/ml streptomycin. Log dilutions of HSA were prepared in 1% NGPS in saline. Appropriate amounts of HSA, PPD, or diluent were added to duplicate or triplicate cultures. Following 48 hr incubation in 5% CO₂-95% air, 4 μCi ^3H-thymidine was added to each culture. After an additional 24 hr of culture, cells were harvested by centrifugation at 1000 rpm for 15 min at 4°C, washed twice in cold saline, and the pellet frozen and thawed three times. Cold 10% trichloracetic acid was added and the precipitate held overnight at 4°C. Tubes were centrifuged at 1500 rpm for 15 min at 4°C and pellets dissolved in Hyamine hydroxide — 10 X (Packard Instrument Co., Inc., Downers Grove, Ill.) at 37°C. Solubilized cultures were transferred quantitatively to Bray's solution and radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. Activities were converted to disintegrations per minute (dpm) by reference to an external standard.

In preliminary experiments HSA nonspecifically depressed thymidine incorporation of cells from animals immunized with CFA alone. This depression was dose dependent, varied in extent with different lots, and was not eliminated by removal of lipid, endotoxin, or the low polymers of albumin known to contaminate commercial preparations (22, 23). The stimulatory response to PPD of cells from animals sensitized with CFA was abolished by the addition of 5 mg/ml HSA whereas 1 mg/ml HSA had no significant effect. While a protein contaminant unique to preparations of HSA could contribute to this suppression (24), a variety of commercially prepared protein antigens including bovine gamma globulin, ferritin, ovalbumin, and keyhole limpet hemocyanin depressed thymidine uptake of CFA-sensitized cells at a concentration of 1 mg/ml, pointing to a less specific toxicity. To minimize the effect of nonspecific toxicity, the response of sensitized or tolerant cells was compared to that of cells from animals receiving adjuvant alone.

Analysis of Data.—Statistical analysis of our data was performed with the kind assistance of Dr. John Gilbert. The mean of dpm for duplicate or triplicate cultures incubated with each concentration of antigen was divided by the mean of dpm incorporated by cells from the same animal grown in the absence of HSA. Ratios thus obtained from 5 to 12 sensitized or tolerant animals were compared to ratios from similar numbers of control animals immunized only with CFA or CFA 1:10. Differences between experimental and control groups were evaluated at each interval and for each concentration of antigen using the Mann-Whitney test (25).
A nonparametric method was chosen, as the variance of ratios from experimental and control groups differed significantly, prohibiting the use of the more familiar t test. Statistically significant stimulation \((P < 0.05)\) is indicated in Figs. 1 and 2 by the use of closed symbols.

For graphical presentation, reactivity was expressed as a stimulatory index. This was calculated for each time interval and antigen concentration by dividing the average ratio of stimulation in each experimental group by the average ratio in the corresponding control group sensitized with adjuvant alone:

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\text{stimulatory index} = \frac{\text{dpm, HSA-CFA-sensitized cells cultured with HSA}}{\text{dpm, HSA-CFA-sensitized cells cultured without HSA}} \cdot \frac{\text{dpm, CFA-sensitized cells cultured with HSA}}{\text{dpm, CFA-sensitized cells cultured without HSA}}
\]

Under favorable circumstances it was possible to detect significant antigen-induced stimulation with stimulatory indices of less than 2 (Figs. 1 and 2).

**Fig. 1.** Stimulation of thymidine incorporation in draining lymph node cells cultured with different concentrations of antigen at 1, 2, and 4 wk after sensitization with 10 \(\mu\)g HSA in CFA. Each point here and in Fig. 2 represents the mean stimulatory index determined from 5–12 guinea pigs sensitized with HSA in CFA, and from a comparable number of animals sensitized with CFA alone (see Materials and Methods). Each closed circle designates a statistically significant difference \((P < 0.05)\) between antigen-sensitized and adjuvant control groups.
Fig. 2. Stimulation of thymidine incorporation in draining lymph node cells cultured with different concentrations of HSA at 2, 4, and 8 wk after sensitization with or without an intravenous suppressing injection: ○—○, Cells from animals sensitized with 10 μg HSA in CFA or CFA 1:10, as indicated. □—■, Cells from animals receiving a 5 mg suppressing injection of HSA at the time of sensitization. △—▲, Cells from animals receiving a 500 μg suppressing injection of HSA at the time of sensitization. Each closed symbol designates a statistically significant difference (P < 0.05) from the adjuvant control group.
Antigen Carry-Over.--At the time of sacrifice, a certain amount of HSA delivered with the sensitizing and/or suppressing injections remained within draining lymph nodes. Since the response of cultured cells proved to vary directly with antigen concentration, it was important to demonstrate that significant quantities of antigen were not transferred to cultures with washed cells. All animals were placed on drinking water containing potassium iodide several days before injection. Five animals were sensitized with 10 μg ¹²⁵I trace-labeled HSA (26) in CFA and another five received a 5 mg suppressing injection of ¹⁰¹I HSA at the time of sensitization with 10 μg unlabeled HSA in CFA. In each case, ¹²⁵I HSA with a specific activity of 200 μCi/mg was diluted with appropriate amounts of unlabeled HSA. 2 wk later, lymph nodes were minced, cells were washed according to the tissue culture protocol, and counted in a Packard autogamma spectrometer.

An average of 4.5 ng HSA was associated with the washed lymph node cells of each animal sensitized with 10 μg labeled HSA in CFA. Less than 1 ng HSA was bound following an intravenous suppressing injection of labeled HSA at the same interval. Assuming that these accumulations are additive, the washed lymph node cells from a single animal receiving both suppressing and sensitizing injections would contain 5.5 ng HSA. Since each culture contained less than 1/40th of the viable cells harvested from an animal, a maximum of 0.2 ng of antigen would have been transferred to any 2.5 ml culture, producing at most an increment of 10⁻⁴ μg HSA/ml. As this was one-thousandth of the lowest antigen concentration employed, the amount of cell-bound antigen transferred should not have affected the results.

RESULTS

Immunologic Response of Sensitized and Tolerant Animals.—The immunologic response of animals receiving toleragenic and/or sensitizing injections of HSA are presented in Table I and are similar to those reported in previous studies (20). Delayed skin reactivity was established at 1 wk in all animals sensitized with 10 μg HSA in CFA. The diameter of cutaneous erythema was maximal at 1 wk, induration maximal at 2 wk, and necrosis apparent in a majority of lesions at 4 wk after immunization. Intravenous administration of 5 mg HSA immediately before sensitization with antigen in adjuvant abolished the skin test response to HSA at 2 wk. Faint cutaneous erythema without induration was elicited at 4 wk, and small, minimally indurated lesions were observed at 8 wk. Suppression of cutaneous reactivity with 500 μg HSA was less complete. Nonindurated erythematous reactions appeared at 2 wk and slightly indurated reactions comparable in diameter to controls developed at 4 wk. However, induration failed to achieve control levels, even at the 8 wk interval. Necrosis was not observed in reactions occurring in partially tolerant animals.

Histologically, characteristic delayed lesions were produced in sensitized animals at 2 and 4 wk, with extensive mononuclear infiltrates throughout the dermis, subadjacent fat, and muscle. Test sites from fully tolerant animals lacked a significant cellular infiltrate (21). Erythematous, nonindurated reactions observed in animals recovering from tolerance, obtained 2 wk after a 500 μg suppressing injection or 4 wk after a 5 mg one, exhibited characteristic cutaneous basophil hypersensitivity (CBH) with a prominent infiltrate of
basophilic leukocytes in addition to mononuclears. CBH has been observed at early intervals after sensitization with antigens in either Freund's complete or Freund's incomplete adjuvant (27), but has not been noted previously in animals recovering from tolerance. At late intervals, the moderately indurated reactions observed on further recovery were similar histologically to the delayed

### TABLE I

**Immunologic Response of Sensitized and Tolerant Animals at Different Intervals Following Immunization with 10 μg HSA**

| Interval | Adjuvant | Tolerating injection of HSA | Delayed skin reactions* | Antibody Titer$ |
|----------|----------|-----------------------------|-------------------------|-----------------|
|          |          | 6 μg HSA | 1:100 OT | | TCA | PCA | TCH |
| wk       |          |          |          | | positive | negative | undiluted | diluted | |
| 1        | CFA      | 0        | 19+(12/12) | 13+(12/12) | 0 (2/6) | 11/12 | 1/12 | 0/12 | 0 (0/6) |
| 2        | CFA      | 0        | 20+++ (13/13) | 18+++ (13/13) | 320 (9/9) | 9/9 | 1/9 | 1/9 | 320 (9/9) |
| 2        | CFA      | 500 μg   | 15+(6/5) | 18+++ (5/5) | 20 (8/12) | 4/12 | 5/12 | 10/12 | 10 (5/12) |
| 2        | CFA      | 5 mg     | 2+(2/14) | 18+++ (14/14) | 20 (6/12) | 10/12 | 5/12 | 10/12 | 0 (0/12) |
| 2        | CFA 1:10 | 0        | 21+++ (6/6) | 17+++ (6/6) | 1250 (6/6) | 5/6 | 0/6 | 5/6 | 1/6 | 640 (6/6) |
| 4        | CFA 1:10 | 0        | 19+++ (10/10) | 24+++ (10/10) | 320 (13/13) | 9/13 | 1/13 | 1/13 | 320 (13/13) |
| 4        | CFA      | 500 μg   | 18+++ (19/19) | 23+++ (19/19) | 80 (10/10) | 9/10 | 1/10 | 1/10 | 40 (8/10) |
| 4        | CFA      | 5 mg     | 6+(6/9) | 22+++ (9/9) | 10 (5/9) | 5/9 | 5/9 | 5/9 | 0 (0/9) |
| 8        | CFA      | 0        | 20+++ (7/7) | 22+++ (7/7) | 160 (7/7) | 1/7 | 4/7 | 2/7 | 0 (0/7) |
| 8        | CFA      | 500 μg   | 18+++ (9/9) | 20+++ (9/9) | 160 (10/10) | 10/10 | 10/10 | 10/10 | 40 (10/10) |
| 8        | CFA      | 5 mg     | 9+(2/3) | 19+++ (3/3) | 0 (0/1) | 1/1 | 1/3 | 1/3 | 0 (0/1) |

* Skin reactions at 24 hr, expressed as average diameter of erythema (mm) and mean degree of induration (0-++++). N indicates necrosis. Numbers in parentheses are fractions giving detectable responses.

$ TCA$, tanned cell hemagglutination, and TCH, tanned cell hemolysis. TCA and TCH expressed as reciprocal of titer. 0 is negative at a serum dilution of 1:5. Numbers in parentheses are fractions of animals with detectable titers. PCA is expressed as the fraction of animals responding at different serum dilutations.

Tuberculin reactivity increased progressively with maximal erythema at 4, and maximal induration at 8 wk. Necrosis was present at both of these intervals. Delayed cutaneous reactivity to tuberculin was not affected by suppressing injections of HSA.

Though antibody could not be detected in sensitized animals at 1 wk by any of the methods employed, including active systemic anaphylaxis, titers were maximal at 2 wk and remained elevated for at least 8 wk. A 5 mg intravenous dose abolished γ₂ production and suppressed γ₁ in nearly all animals for the
duration of the experiment. With a 500 \( \mu g \) suppressing injection, hemagglutination titers were significantly reduced, though not eliminated, at 2 and 4 wk, and eventually rose to control levels. PCA activity exhibited a similar pattern and actually exceeded control values at 8 wk. Hemolytic antibody remained depressed at all intervals.

**Antigen-Induced Thymidine Uptake by Cells from Sensitized Animals.**—Groups of from 5–12 guinea pigs were immunized with 10 \( \mu g \) HSA in CFA or with CFA alone and cultures were prepared at 1, 2, 4, and 8 wk thereafter. Incorporation of tritiated thymidine by lymph node cells was measured in the absence or presence of different concentrations of HSA ranging from \( 10^{-1} \) to \( 10^{+4} \mu g/ml \). The effect of 10 \( \mu g \) PPD/ml was also studied.

At 1 wk after immunization, when delayed cutaneous reactivity was well established and antibody not yet demonstrable, a statistically significant \( (P = 0.009) \) increase of thymidine incorporation could be achieved with 10 mg HSA/ml (Fig. 1). At subsequent intervals, cells failed to react with 10 mg HSA/ml but significant stimulation could be produced with as little as 100 \( \mu g/ml \) at 2 wk and 0.1 \( \mu g/ml \) at 4 and 8 wk (Figs. 1 and 2). Similarly, 2-fold stimulation occurred at progressively lower concentrations of HSA, requiring 10 mg/ml at 1 wk and only 10 \( \mu g/ml \) at 4 wk. Over the same interval the maximal stimulatory index increased from 2.4 at 1 wk to 6.6 at 4 wk.

10 \( \mu g \) PPD/ml produced 4-, 5-, and 4-fold stimulation on the average at 2, 4, and 8 wk respectively.

**Antigen-Induced Thymidine Uptake by Cells from Tolerant Animals.**—Lymph node cells from animals rendered tolerant with 500 \( \mu g \) or 5 mg HSA were cultured 2, 4, and 8 wk after sensitization and their responses were compared with those of cells from animals sensitized with HSA in adjuvant or with adjuvant alone (Fig. 2).

A 5 mg intravenous injection of HSA administered at the time of sensitization eliminated an in vitro response to specific antigen at 2 wk over the entire range of concentrations tested, correlating with the complete suppression of cutaneous reactivity and antibody production observed in vivo. By 4 wk significant stimulation could be achieved, but only with 1 mg HSA/ml. At this dose level the magnitude of response was significantly reduced relative to that of cells from sensitized animals that had not received a suppressing injection. Return of reactivity at high antigen concentration in vitro was associated with the appearance of a nonindurated CBH reaction while neither \( \gamma_1 \) nor \( \gamma_2 \) antibody could be detected. At 8 wk, the magnitude of response with 1 mg HSA/ml could no longer be distinguished statistically from that of sensitized animals. However, cells from tolerant animals failed to react at all lower antigen concentrations.

Suppression of lymphocyte stimulation varied with the dose of antigen administered. Following 500 \( \mu g \) of HSA, tolerance was less complete and re-
covery more rapid than that observed after the larger suppressing dose. Significant stimulation was observed during the 2nd wk at 1 mg/ml, though not at lower antigen concentrations. The magnitude of response exhibited by sensitized cells was significantly greater at both 100 μg and 1 mg/ml. By 4 wk the response of sensitized and tolerant cells to 1 mg HSA/ml could no longer be distinguished statistically. Significant reactivity was present at HSA concentrations as low as 10 μg/ml, indicating a progressive, though partial, recovery of response to moderate concentrations of antigen. Reactivity persisted at high antigen concentration for at least 8 wk, but sensitized cells still proved significantly more reactive at 0.1–10 μg/ml, consistent with the conclusion that reactivity had been suppressed at low antigen concentration throughout the experiment. It may be significant that the induration of cutaneous reactions and hemolytic antibody titers also failed to achieve control levels in the 500 μg tolerant group, while hemagglutinating and PCA titers equaled or exceeded those of sensitized controls.

Cells from all tolerant groups responded to 10 μg PPD/ml with stimulation comparable to that observed in cells sensitized with HSA in CFA or with CFA alone, indicating that the suppression was antigen specific.

**Effect of Mycobacteria on Sensitization and Induction of Tolerance.**—Guinea pigs sensitized with 10 μg HSA in Freund’s complete adjuvant containing 300 μg tubercle bacilli/ml (CFA 1:10) developed delayed cutaneous reactivity and antibody titers entirely comparable to those of animals immunized with the adjuvant usually employed (Table I). However, administration of a 5 mg intravenous injection at the time of sensitization failed to produce the complete suppression of cutaneous reactivity observed at 2 wk in animals sensitized with adjuvant containing the larger dose of tubercle bacilli.

When studied in vitro (Fig. 2), cells from animals sensitized with HSA in CFA 1:10 2 wk earlier could be stimulated with 10 mg HSA/ml while cells from guinea pigs immunized with HSA in CFA failed to react at this high concentration of antigen. Both groups responded to 10 and 100 μg HSA/ml. Following a 5 mg suppressing injection, lymphocyte stimulation was abolished in animals immunized with HSA in CFA, but could be elicited with 100 μg–10 mg HSA/ml in the CFA 1:10 groups, correlating with the cutaneous reactions observed in vivo.

**DISCUSSION**

Data presented here document an evolution of the cellular immune response consistent with the selection of lymphoid cells by exposure to decreasing concentrations of antigen in vivo (7). Within 1 wk of sensitization with HSA in CFA, cells appeared in draining lymph nodes which were capable of being stimulated in vitro with 10 mg HSA/ml. By 4 wk reactivity had disappeared at this high antigen concentration, but statistically significant stimulation
could be produced over a 10,000-fold range from 1 mg/ml to 0.1 μg/ml. Loss of reactivity at 10 mg/ml may reflect an early decrease in the concentration of immunizing antigen below a threshold required to prime cells of the “lowest affinity,” associated with depletion of primed cells, through migration or death. Actual measurement of antigen accumulation in draining nodes after sensitization with HSA in CFA has demonstrated maximal antigen levels at 1 wk with a subsequent rapid decline (28). Cells reacting at low antigen concentration appeared only at later intervals when the concentration of HSA in vivo had fallen, presumably permitting the selective proliferation or recruitment of “higher affinity” cells. After 4 wk, reactivity was not observed at concentrations below 0.1 μg/ml and no further increase occurred in the magnitude of maximal stimulation. Attainment of an optimal cellular response by 4 wk may explain the inability of Paul et al. (10) to detect differences in the reactivity of cells cultured at 7–19 days and at 5–7 months.

Interpretation of our data in terms of an affinity model is complicated by the use of HSA, a protein antigen bearing multiple determinants. In our system development of reactivity at low antigen concentration might represent the cellular response to strong or abundant determinants, rather than the response of cells with high affinity for any single antigenic group. Similarly, the response at high antigen concentration could reflect stimulation of cells bearing receptors for weak or less abundant determinants of different specificities, rather than the contribution of cells of low affinity. In either event, the appearance with time of in vitro reactivity at progressively lower antigen concentration would reflect the antigen-mediated selection of reactive cells from a population heterogeneous with regard to specificity for different determinants, affinity for individual determinants, or both. For a variety of conditions our data have established the minimum antigen concentration at which cells are stimulated in culture; however, they afford no insight into the behavior of cells at suprathreshold levels of antigen. Thus, the greater stimulatory indices observed at high antigen concentrations might reflect the presence in lymph nodes of a large population of cells reactive only at high antigen concentrations; alternatively, if cells continue to respond at antigen concentrations well above the minimum needed for stimulation, these indices might reflect a summation of the proliferative activities of all cells whose threshold was exceeded.

Lymphocytes derived from animals rendered tolerant by a suitable injection of soluble antigen at the time of sensitization were not stimulated by specific antigen in culture. This confirms earlier reports that cells from tolerant animals remain specifically incompetent in vitro or on transfer to irradiated hosts (29–33). Suppression of lymphocyte stimulation by an intravenous injection of antigen proved to be a dose-related phenomenon exhibiting kinetics compatible with a selection of cells by antigen. Administration of 5 mg HSA prevented lymphocyte stimulation for at least 2 wk at all antigen concentra-
tions employed in culture, while 500 μg HSA eliminated stimulation at low, but not at the highest antigen concentrations over this interval. Partially tolerant animals or animals beginning to recover from tolerance possessed lymph node cells responsive to high antigen concentrations and developed CBH (27). With time, reactivity at lower HSA concentrations was also observed. Similarities of the dose response curves at 4–8 wk after a 5 mg suppressing injection, 2–4 wk after 500 μg, or 1–2 wk after sensitization alone, suggest that the evolution of reactivity observed after administration of antigen in adjuvant may simply be delayed in tolerant animals. However, recapitulation of the primary response was not complete in that reactivity did not return at the lowest antigen concentrations during the course of our study. This selective loss would be predicted if cells reacting at low antigen concentrations bound HSA most avidly and if such binding enhanced the toleragenic effect of antigen. Similarly, failure to induce complete tolerance in animals sensitized with HSA in CFA 1:10 reflects the presence in such animals of large numbers of cells reactive only at high antigen concentrations, the cell population most difficult to render tolerant. The cellular kinetics observed are therefore reminiscent of the observations of Theis and Siskind (4) who found that the small amount of antibody formed by partially tolerant rabbits was of low average affinity and that affinity gradually rose with recovery from tolerance, eventually achieving values attained by normal animals much earlier in the course of sensitization. Similar data have been obtained in studies of antibody produced by tolerant animals sensitized with cross-reacting antigens (34).

Extrapolations from studies of lymphocyte stimulation to an understanding of the heterogeneity of the humoral response are limited by the finding that antigen-induced thymidine incorporation correlates temporally with delayed cutaneous reactivity rather than with elevations of antibody titer. Lymphocyte stimulation was produced at 1 wk after sensitization when cutaneous reactivity was well established in the absence of detectable antibody, confirming the report of Oppenheim in a hapten system (18). The appearance of lymphocyte reactivity 4 wk after a 5 mg suppressing injection correlated with the development of erythematous nonindurated skin reactions exhibiting the histology of CBH (27). Only minimal titers of hemagglutinating antibody were present at this time, hemolytic antibody could not be detected, and only one of nine sera had PCA activity. Histologically, the lymphocytes and blasts encountered in these cultures contained little endoplasmic reticulum and did not resemble plasma cell precursors. While antibody can be produced by cells resembling typical lymphocytes, only small amounts of antibody have been detected in similar cultures despite numerous studies (35). On the other hand, migration-inhibitory factor, thought to be a mediator of delayed reactions in vivo, is readily produced by lymph node cells in the presence of specific antigen (36).

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2 Dvorak, A. M., R. C. Bast, Jr., and H. F. Dvorak. Unpublished data.
Taken together, these observations point to an association of lymphocyte stimulation in culture with cell-mediated events in vivo, a conclusion that will be discussed in the following paper (28).

Insofar as lymphocyte stimulation correlates with cell-mediated immunity, our data suggest one explanation for the relative ease with which tolerance to delayed reactivity can be produced in adult animals by a single injection of antigen (20). Standard delayed skin tests of 6 μg in 0.1 ml provide an initial local concentration of HSA no greater than 60 μg/ml. More than 50% of this dose is cleared from the test site by 6 hr when mononuclears begin to accumulate, and more than 90% of injected antigen is cleared within 24 hr (21). Concentrations of antigen in situ, then, correspond to levels that stimulate cells reacting at relatively low antigen concentration in culture and these are precisely the cells most readily eliminated by the intravenous injection of antigen. In fact, stimulation was not observed at antigen concentrations of less than 10 μg/ml in animals recovering from tolerance. If cells reacting at lower HSA levels contribute significantly to delayed lesions, their selective loss would explain the failure of tolerant animals to achieve indurated cutaneous reactions comparable to controls.

Finally, it is worth noting that a 5 mg suppressing injection provides an adult guinea pig with initial plasma levels of approximately 250 μg HSA/ml. After 24–48 hr this value falls to 25–50 μg/ml through equilibration with the extracellular fluid, catabolism, and excretion. Excluding the possibility of a mechanism enhancing the local concentration of antigen by some 1000-fold, animals are rendered tolerant in vivo with concentrations of antigen producing lymphocyte stimulation in culture. These figures may help to explain the difficulty experienced by many workers in attempts to induce tolerance to heterologous serum proteins by exposure to antigen in vitro (30). Possibly an additional step of complexity is provided within the whole animal beyond the simple interaction of lymphocytes with antigen. Alternatively, lymphocyte proliferation resembling that observed in culture might constitute a critical step in the development of tolerance.

SUMMARY

Lymph node cells from guinea pigs immunized with HSA in complete Freund's adjuvant were grown in cultures containing different concentrations of specific antigen. Stimulation of thymidine incorporation was induced with progressively lower concentrations of HSA at successive intervals after sensitization. Moreover, the intensity of delayed skin reactions and the magnitude of stimulation in vitro increased over the same interval. These events are considered compatible with an evolution of the cellular immune response resulting from the selection of lymphoid cells by decreasing concentrations of antigen in vivo.
Cells from animals rendered tolerant to HSA failed to respond to specific antigen in culture. As tolerance waned, stimulation was achieved at high but not low antigen concentrations. Tolerance, measured by cutaneous reactivity or by lymphocyte stimulation, was less readily induced in animals sensitized with adjuvant containing a reduced concentration of mycobacteria. Lymph nodes from these animals contained a large population of cells reactive at high antigen concentration, presumably less susceptible to the toleragenic effect of intravenous antigen.

The dissociation of delayed hypersensitivity and antibody formation observed early in the immune response and upon recovery from tolerance has permitted correlation of lymphocyte stimulation with delayed hypersensitivity and cutaneous basophil hypersensitivity respectively.

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