

## HETEROGENEITY OF THE CELLULAR IMMUNE RESPONSE

### II. THE ROLE OF ADJUVANT. LYMPHOCYTE STIMULATION IN CUTANEOUS BASOPHIL HYPERSENSITIVITY\*

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Modification of the cellular immune response by sensitization with adjuvants containing tubercle bacilli has been carefully studied in whole animals of several species (1-5). Guinea pigs immunized with a foreign protein in Freund's complete adjuvant (CFA)<sup>1</sup> develop persistent delayed hypersensitivity with an erythematous skin test response that is delayed in onset, indurated, and characterized histologically by a prominent mononuclear infiltrate (1, 6). By contrast, animals immunized with a protein in Freund's incomplete adjuvant (IFA) without tubercle bacilli exhibit an erythematous reaction that is delayed in onset, but that lacks significant induration and that can only be elicited at early intervals after sensitization (7-10). Raffle and Newel (7) differentiated this "Jones-Mote" reaction from that of classic delayed hypersensitivity, but many have considered Jones-Mote hypersensitivity only a weak expression of the delayed response (see reference 11 for review).

Recently, Dvorak et al. (10) have demonstrated that Jones-Mote lesions exhibit a distinct histology, featuring substantial accumulations of basophilic leukocytes in addition to mononuclear cells, and have suggested that cutaneous basophil hypersensitivity (CBH) would provide a more descriptive name for this reaction. CBH resembles delayed hypersensitivity in that both can be transferred passively with sensitized lymph node cells, but not with serum<sup>2</sup> (12), both require an immunogenic protein or protein conjugate for elicitation (9), and both are inhibited by anti-lymphocyte serum (9).

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<sup>1</sup> *Abbreviations used in this paper:* CBH, cutaneous basophil hypersensitivity; CFA, complete Freund's adjuvant; HSA, human serum albumin; IFA, incomplete Freund's adjuvant; MEM, Eagle's minimal essential medium; MIF, migration-inhibitory factor; NGPS, normal guinea pig serum; OT, old tuberculin; PCA, passive cutaneous anaphylaxis; PPD, preservative-free purified protein derivative.

<sup>2</sup> Dvorak, H. F., R. C. Bast, Jr., and S. Leskowitz. Unpublished data.

In the present study we compare the proliferative response of cells isolated from guinea pigs exhibiting either CBH or delayed hypersensitivity. Lymph node cells from animals primed for CBH with human serum albumin (HSA) in IFA exhibited striking stimulation of thymidine incorporation on exposure to specific antigen. The *in vitro* response, like the associated skin reaction, was transient and could be elicited only at early intervals after sensitization. A persistent proliferative response occurring at progressively lower antigen concentration (13) was attained after immunization with the same amount of HSA in emulsions containing tubercle bacilli. In addition, antigen-specific inhibition of macrophage migration was demonstrated only with exudates from animals sensitized with complete adjuvant. In an attempt to explain these differences in response, tissue concentrations of antigen were measured following immunization with HSA emulsified in complete and incomplete adjuvants.

#### *Materials and Methods*

*Antigens and Adjuvants.*—Solutions of HSA, crystallized, old tuberculin (OT), and preservative-free purified protein derivative of tuberculin (PPD)<sup>3</sup> were prepared as previously described (13).

CFA contained 50 parts saline, 42.5 parts Bayol 55, 7.5 parts Arlacel A,<sup>4</sup> and sufficient heat-killed tubercle bacilli to provide a final concentration of 3 mg/ml. In some experiments the concentration of tubercle bacilli was reduced 10-fold to 300  $\mu$ g/ml (CFA 1:10). IFA was prepared from the same formula, omitting tubercle bacilli.

*Immunizations, Skin Tests, and Antibody Assays.*—Male Hartley guinea pigs (450–500 g) were sensitized with 100  $\mu$ g HSA emulsified in IFA, CFA, or CFA 1:10, divided equally among the four footpads. Control groups received adjuvant lacking HSA. Tolerance was induced with 5 mg HSA in 1 ml saline injected intravenously immediately before sensitization.

Skin tests were performed with 10 or 50  $\mu$ g HSA and with 1:100 or 1:500 OT. Reactions were scored and examined histologically as previously described (10). Anti-HSA titers were determined by tanned red cell hemagglutination and hemolysis, and by passive cutaneous anaphylaxis (PCA) (13).

*Lymphocyte-Culture and Macrophage-Migration Studies.*—Lymphocyte cultures were prepared and data analyzed by the methods described in the preceding paper (13).

For studies of macrophage migration, the technique of David et al. (14) was employed. Exudates were obtained from sensitized or nonsensitized Hartley guinea pigs 4 days after intraperitoneal injection of 20 ml of sterile Bayol 55, washing the peritoneum with 150 ml of Hanks' balanced salt solution. Cells were concentrated by centrifugation at 1000 rpm for 10 min at 4°C, washed twice with Hanks' solution, and resuspended to 10% by volume in Eagle's minimal essential medium (MEM) supplemented with 15% fresh normal guinea pig serum (NGPS). Log dilutions of HSA were prepared in saline supplemented with 1% NGPS. Appropriate amounts of antigen or diluent were added to samples of the cell suspension. Capillary tubes were filled from these samples, sealed, and centrifuged at 800 rpm for 5 min. Capillaries were cut at the cell fluid interface, and secured in a Mackness chamber with a small amount of silicone grease. Chambers were sealed with paraffin and filled with MEM containing 15% NGPS, 100 units/ml of penicillin, 100  $\mu$ g/ml of streptomycin, and appropriate amounts of

<sup>3</sup> The generous gift of Parke, Davis & Co., Inc., Detroit, Mich.

<sup>4</sup> For sources of these and the following materials see preceding article.

antigen or diluent. Two capillaries were positioned in each chamber and four chambers prepared for each concentration of antigen (0, 10, 100, and 1000  $\mu\text{g}/\text{ml}$  HSA). Following incubation for 24 hr at 37°C in 5%  $\text{CO}_2$ -95% air, areas of migration were projected, traced, and measured by planimetry. Cytotoxic indices were calculated for cells from each animal:

$$\text{cytotoxic index} = \frac{\text{average area of migration with antigen}}{\text{average area of migration without antigen}}$$

Average cytotoxic indices were calculated for each group of four to six animals and the significance of differences between groups was evaluated with the *t* test.

*Antigen Carry-Over.*—To estimate the amount of antigen transferred to cultures with lymph node cells from sensitized animals, groups of five guinea pigs were immunized with 100  $\mu\text{g}$   $^{125}\text{I}$  trace-labeled HSA (specific activity 100  $\mu\text{Ci}/\text{mg}$ ) in CFA or IFA (13). Animals were placed on potassium iodide several days before injection. 1 wk after sensitization, draining lymph nodes were harvested, processed for tissue culture, and counted in a gamma spectrometer. Estimated increments of  $4 \times 10^{-4}$  and  $8 \times 10^{-4}$   $\mu\text{g}$  HSA/ml were contributed to each culture by  $1 \times 10^7$  cells sensitized with HSA in IFA and CFA respectively. As these values represent less than 1% of the minimal antigen concentration employed to induce lymphocyte stimulation, the amount of antigen carried over would not have affected our experimental results.

*Release and Systemic Distribution of Antigen in Different Adjuvants.*—In order to determine the rate of release of HSA from adjuvant depots and the fate of disseminated antigen, groups of five guinea pigs treated with potassium iodide were sensitized with 100  $\mu\text{g}$   $^{125}\text{I}$  HSA (4.1  $\mu\text{Ci}$ ) incorporated in CFA, CFA 1:10, or IFA. At various intervals after immunization, animals were exsanguinated and different tissues excised and counted in a Packard autogamma spectrometer (Packard Instrument Co., Inc., Downer's Grove, Ill.) Four feet, spleen, thymus, a single femur, plasma, and the draining lymph nodes were removed from each animal. Lymph nodes were weighed to permit calculation of specific activity.

## RESULTS

*Immunologic Response to HSA Administered with Different Adjuvants.*—Animals immunized with 100  $\mu\text{g}$  HSA in IFA exhibited erythematous, nonindurated, delayed onset skin reactions characteristic of CBH at 1 and 2 wk after sensitization (Table I). At 4 and 6 wk, reactions achieved maximal intensity in a few hours, but persisted at 24 hr as slightly indurated lesions of one-half the diameter observed in animals skin tested at earlier intervals. These "late" reactions differ from CBH histologically and may be mediated by antibody (10). Immunization with 100  $\mu\text{g}$  HSA in either CFA or CFA 1:10 produced characteristically indurated skin-test reactions of comparable diameter at 1 wk. Reactions were more indurated, though no larger, at 6 wk.

Hemagglutinating antibodies were present in high titer at 1 and 6 wk in all groups sensitized with 100  $\mu\text{g}$  HSA. However, animals sensitized with IFA produced significant titers of PCA ( $\gamma_1$ ) but not of hemolytic ( $\gamma_2$  and macroglobulin) antibody at 1 wk. With CFA, low titers of hemolytic antibody were observed in the absence of PCA activity. With CFA 1:10, low titers of both types of antibody were produced at 1 wk. By 6 wk, significant titers of PCA and hemolytic antibody were elicited with all three adjuvants.

*Effect of Sensitization with Different Adjuvants on Antigen-Induced Thymidine Uptake.*—Groups of 6–14 guinea pigs were immunized with 100  $\mu$ g HSA in IFA, CFA, or CFA 1:10, and equivalent groups were sensitized with the various adjuvants lacking HSA. Cultures of draining lymph node cells were prepared at 1 and 6 wk and incorporation of  $^3$ H-thymidine was measured as a function of HSA concentration in vitro.

TABLE I  
*Immunologic Response at Different Intervals in Animals Sensitized with 100  $\mu$ g HSA in Various Adjuvants*

| Interval  | Adjuvant  | Tolerizing injection of HSA | Delayed skin reactions*   |                          | Antibody Titers† |           |             |              |                |
|-----------|-----------|-----------------------------|---------------------------|--------------------------|------------------|-----------|-------------|--------------|----------------|
|           |           |                             | 50 $\mu$ g HSA            | 10 $\mu$ g HSA           | TCA              | PCA       |             | TCH          |                |
|           |           |                             |                           |                          | Negative         | Undiluted | $\geq 1:20$ | $\geq 1:100$ |                |
| <i>wk</i> | <i>mg</i> |                             |                           |                          |                  |           |             |              |                |
| 1         | CFA       |                             | 23 <sup>++</sup> (6/6)    | 16 <sup>+</sup> (9/10)   | 640 (8/8)        | 8         | 0           | 0            | 20 (6/8)       |
|           | CFA 1:10  |                             | 23 <sup>++</sup> (6/6)    | 15 <sup>+</sup> (5/6)    | 1280 (6/6)       | 2         | 1           | 3            | 20 (5/6)       |
|           | IFA       |                             | 17 <sup>0</sup> (8/8)     | 12 <sup>0</sup> (11/11)  | 320 (14/14)      | 3         | 3           | 7            | 0 (0/14)       |
|           | IFA       | 5                           | 5 <sup>0</sup> (4/6)      | 2 <sup>0</sup> (3/6)     | 40 (3/5)         | 5         | 0           | 0            | 0 (0/5)        |
| 2         | IFA       |                             | 20 <sup>0</sup> (6/6)     | 9 <sup>0</sup> (6/6)     | 640 (6/6)        | 0         | 0           | 1            | 5 80 (6/6)     |
|           | IFA       | 5                           | 20 <sup>0</sup> (5/6)     | 12 <sup>0</sup> (5/6)    | 80 (5/6)         | 2         | 1           | 3            | 0 0 (0/6)      |
| 4         | IFA       |                             | 12 <sup>+</sup> (6/6)     | 9 <sup>+</sup> (5/6)     | 640 (11/11)      | 0         | 0           | 0            | 11 320 (11/11) |
|           | IFA       | 5                           | 22 <sup>+</sup> (6/6)     | 12 <sup>0</sup> (6/6)    | 640 (11/11)      | 0         | 0           | 6            | 5 10 (3/11)    |
| 6         | CFA       |                             | 22 <sup>+++</sup> (11/11) | 13 <sup>++</sup> (11/11) | 640 (11/11)      | 1         | 1           | 3            | 6 640 (11/11)  |
|           | CFA 1:10  |                             | 21 <sup>+++</sup> (6/6)   | 12 <sup>++</sup> (6/6)   | 1280 (6/6)       | 0         | 0           | 2            | 4 1280 (6/6)   |
|           | IFA       |                             | 11 <sup>+</sup> (7/9)     | 7 <sup>0</sup> (7/9)     | 640 (11/11)      | 0         | 0           | 9            | 2 320 (11/11)  |

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

† TCA, tanned cell hemagglutination; TCH, tanned cell hemolysis. TCA and TCH expressed as reciprocal of titer. 0 is negative at 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 dilutions.

At 1 wk, cells from animals immunized with HSA in IFA showed 6-fold stimulation relative to controls at an optimal antigen concentration of 10 mg/ml (Fig. 1). Statistically significant stimulation was achieved with as little as 10  $\mu$ g/ml. By contrast, cells from animals sensitized with HSA in CFA gave a weak, irregular response at 1 wk, showing significant stimulation only at an antigen concentration of 1 mg/ml. Immunization with HSA in CFA 1:10 led to an intermediate response with optimal stimulation at 1 mg/ml, and a significantly enhanced incorporation at 10  $\mu$ g/ml.

At 6 wk, the pattern of response had reversed (Fig. 2). Cells from animals sensitized with HSA in IFA failed to respond at any antigen concentration tested, whereas sensitization with the same amount of HSA in CFA permitted

significant stimulation with as little as  $0.1 \mu\text{g}$  HSA/ml. An optimal stimulatory index of 5, observed at 1 or 10 mg HSA/ml, was comparable to the maximal index achieved with HSA in IFA at 1 wk.

*Production of Partial Tolerance to CBH.*—A single intravenous injection of 5 mg HSA at the time of sensitization with  $100 \mu\text{g}$  HSA in IFA substantially reduced but did not eliminate cutaneous reactivity at 1 wk (Table I). By 2 wk,

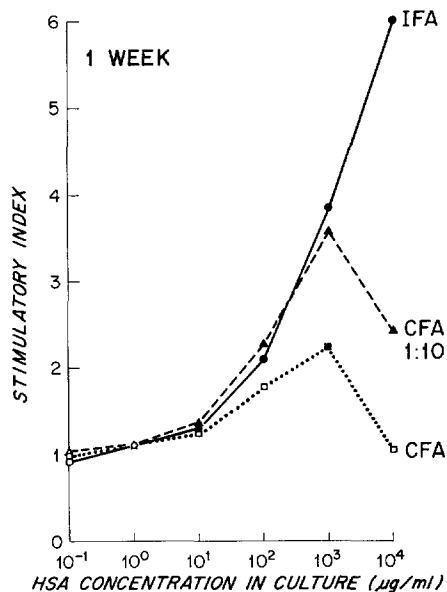


FIG. 1. Stimulation of thymidine incorporation by draining lymph node cells cultured with different concentrations of antigen at 1 wk after sensitization with  $100 \mu\text{g}$  HSA incorporated in IFA, CFA, or CFA 1:10. Each point here and in Figs. 2 and 3 represents the mean stimulatory index determined from 5–12 guinea pigs sensitized with HSA in adjuvant, and from a like number of animals sensitized with each of the adjuvants alone (see Materials and Methods).  $\circ$ — $\bullet$ , IFA;  $\square$ — $\blacksquare$ , CFA; and  $\triangle$ — $\blacktriangle$ , CFA 1:10. A closed symbol designates a statistically significant antigen-induced stimulation ( $P < 0.05$ ) as compared with adjuvant controls.

skin reactions were comparable to, and at 4 wk actually exceeded reactions observed in sensitized controls. Hemagglutination and PCA titers were only transiently depressed but hemolytic antibody was effectively reduced for at least 4 wk.

When cells from partially tolerant animals were cultured at 1 wk after sensitization (Fig. 3), significant stimulation was obtained only at the highest antigen concentration studied (10 mg/ml). Even at this antigen level, the response of tolerant cells was significantly reduced relative to sensitized controls.

*Inhibition of Macrophage Migration.*—Guinea pigs were immunized with  $100 \mu\text{g}$  HSA in either IFA or CFA and peritoneal exudates obtained at 1 and

3 wk. Macrophage migration from capillary tubes was measured in the absence of antigen and in concentrations of HSA ranging from 10 to 1000  $\mu\text{g}/\text{ml}$ . Cytotoxic indices significantly different from those obtained with nonsensitized control exudates were observed only with cells from animals immunized with HSA in CFA and only at 3 wk after sensitization (Table II). At this interval 10  $\mu\text{g}/\text{ml}$  HSA inhibited migration by 50%, and progressively greater inhibition was obtained with higher antigen concentrations. Peritoneal exudate cells

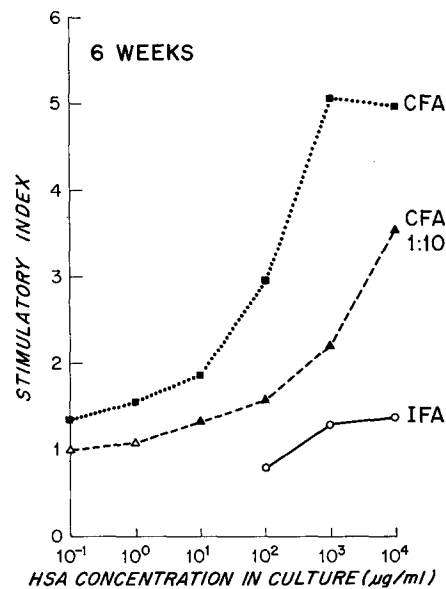


FIG. 2. Stimulation of thymidine incorporation by draining lymph node cells cultured with different concentrations of antigen at 6 wk after sensitization with 100  $\mu\text{g}$  HSA incorporated in IFA, CFA, or CFA 1:10. Symbols same as for Fig. 1.

from animals immunized with HSA in IFA were not inhibited by even the highest antigen concentration at either interval studied.

*Release and Systemic Distribution of Antigen in Different Adjuvants.*—In an attempt to account for the differences observed after sensitization with HSA in different adjuvants, the clearance and distribution of radioactive HSA was studied. Guinea pigs were immunized with 100  $\mu\text{g}$   $^{125}\text{I}$  HSA in IFA, CFA, or CFA 1:10, and various organs were sampled from groups of five animals at intervals up to 6 wk. The results of this study are summarized in Fig. 4 and in Table III.

Antigen was cleared from the footpads exponentially and at similar rates, regardless of the adjuvant employed. Approximately one-third of the depot was exhausted at 1 day, one-half at 4 days, and two-thirds at 1 wk. At 6 wk all groups retained about 10% of injected antigen in their footpads. Similarity

between groups at all intervals was remarkable considering the swelling and gross ulceration of footpads produced by the adjuvants containing tubercle bacilli.

A sharp peak of activity appeared in the plasma, spleen, thymus, and femur

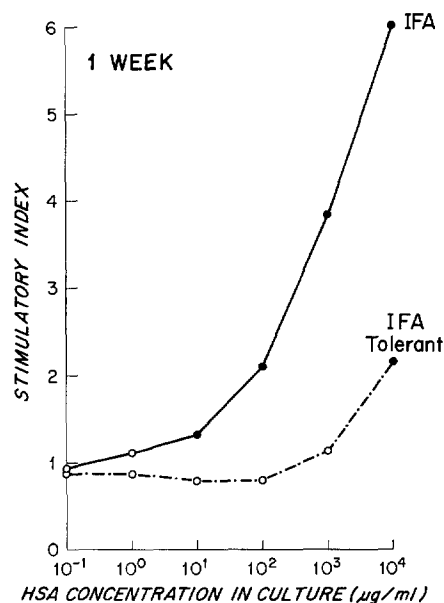


FIG. 3. Stimulation of thymidine incorporation of draining lymph node cells cultured with different concentrations of HSA 1 wk after sensitization with 100 µg HSA in IFA (○—●). IFA-tolerant group (○---●) received an additional suppressing injection of 5 mg HSA at the time of sensitization. Closed circles designate statistically significant stimulation ( $P < 0.05$ ).

TABLE II  
*Inhibition of Macrophage Migration in Delayed Hypersensitivity and Cutaneous Basophil Hypersensitivity*

| Immunization of peritoneal exudate donor | Time after immunization (wk) | Cytotoxic index*                     |             |             |
|--|------------------------------|--------------------------------------|-------------|-------------|
|  |                              | HSA concentration in culture (µg/ml) |             |             |
|  |                              | 10                                   | 100         | 1000        |
| Not immunized                            | —                            | 0.99 ± .08                           | 1.08 ± .06  | 1.31 ± .14  |
| 100 µg HSA-IFA                           | 1                            | 1.11 ± .10                           | 1.05 ± .12  | 1.34 ± .13  |
| 100 µg HSA-CFA                           | 1                            | 1.18 ± .08                           | 1.10 ± .07  | 0.88 ± .07  |
| 100 µg HSA-IFA                           | 3                            | 0.98 ± .04                           | 1.02 ± .08  | 1.02 ± .08  |
| 100 µg HSA-CFA                           | 3                            | 0.50 ± .06‡                          | 0.37 ± .06‡ | 0.30 ± .04‡ |

\* Mean cytotoxic index ± SE. Each number represents the average value obtained from octuplicate cultures prepared from four to six animals.

‡ Statistically significant inhibition as compared with controls ( $P < 0.01$ ).

1 day after sensitization, and declined rapidly over the next week (Fig. 4). At 1 day, animals sensitized with antigen in IFA had ten times the plasma concentration and five times the tissue levels of animals immunized with CFA. Sensitization with CFA 1:10 led to an intermediate accumulation.

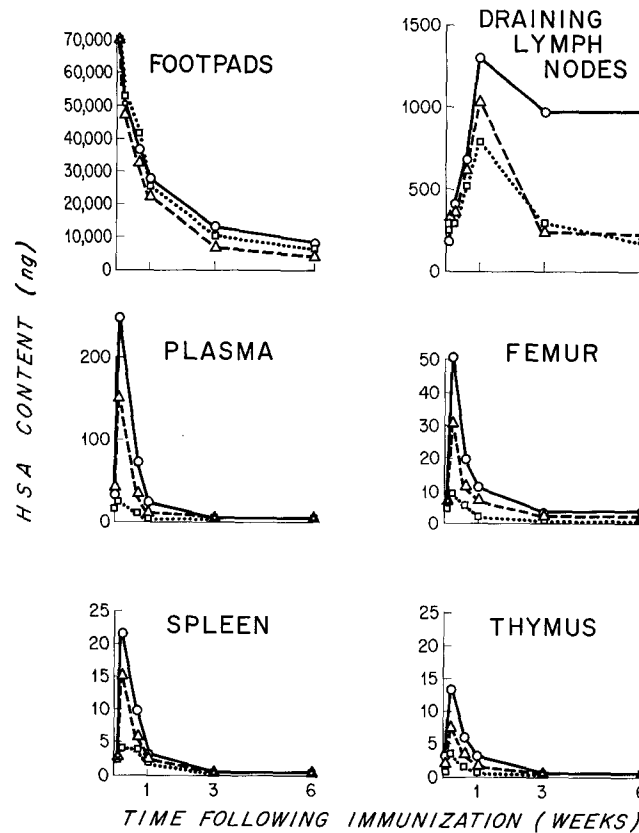


FIG. 4. Distribution of human albumin in various tissues with time after sensitization in the four footpads with  $100 \mu\text{g } ^{125}\text{I}$  HSA in IFA ( $\circ$ — $\circ$ ), CFA ( $\square$ ··· $\square$ ), or CFA 1:10 ( $\triangle$ — $\triangle$ ). Data is expressed as nanograms of HSA in the four footpads, pooled draining lymph nodes, 1 ml of plasma, one femur, entire spleen, and entire thymus. Each point represents the average value obtained from five separate animals.

The concentration of antigen in draining lymph nodes was of particular interest. Antigen continued to accumulate during the 1st wk, and here again a significantly higher antigen concentration was found after IFA immunization. Differences in antigen concentration were most dramatic when specific activities (total HSA/node weight) were compared, since adjuvants containing tubercle bacilli produced significantly larger lymph nodes containing more lymphoid



cells (Table III). Thus, at 1 wk there was a 6-fold increase and at 3 and 6 wk a 10-fold increase in the specific activities of HSA in lymph nodes draining an IFA depot as compared with nodes draining antigen in CFA. Similar values were obtained when specific activity was calculated on the basis of antigen per million recoverable draining lymph node cells (a 3-fold difference at 1 wk and a 13-fold difference at 6 wk) (Table III).

Total antigen retained within draining lymph nodes fell 3–4-fold between 1 and 3 wk in animals sensitized with antigen in CFA or CFA 1:10; disappearance of antigen after IFA sensitization was considerably slower.

TABLE III  
*Concentration of HSA in Draining Lymph Nodes at Various Intervals after Sensitization with 100  $\mu$ g of Trace-Labeled Antigen in CFA, CFA 1:10, and IFA.*

| Time after sensitization | Adjuvant | Total HSA | Node weight* | Specific activity     | Total cells<br>( $\times 10^6$ ) $\ddagger$ | Specific activity                   |
|--------------------------|----------|-----------|--------------|-----------------------|---|-------------------------------------|
|                          |          |           |              | ng HSA<br>Node weight |   | ng HSA $\times 10^6$<br>Total cells |
| (wk)                     |          | (ng)      | (g)          |                       |   |                                     |
| 1                        | IFA      | 1298      | 0.4999       | 2611                  | 270   | 4.8                                 |
|                          | CFA 1:10 | 1022      | 0.8733       | 1490                  | 388   | 2.6                                 |
|                          | CFA      | 781       | 1.7924       | 446                   | 494   | 1.6                                 |
| 3                        | IFA      | 960       | 0.8443       | 1141                  |   |                                     |
|                          | CFA 1:10 | 243       | 1.3072       | 184                   |   |                                     |
|                          | CFA      | 273       | 2.6305       | 106                   |   |                                     |
| 6                        | IFA      | 949       | 0.9293       | 1077                  | 217   | 4.4                                 |
|                          | CFA 1:10 | 180       | 1.4461       | 125                   | 452   | 0.4                                 |
|                          | CFA      | 179       | 1.9273       | 95                    | 516   | 0.35                                |

\* Wet weight.

$\ddagger$  Total cells recovered from all draining lymph nodes.

#### DISCUSSION

Our data indicate that the type of cellular immunity evoked in an animal—its duration, evolution, and particularly the pattern of lymphocyte response in culture—is greatly affected by the concentration of tubercle bacilli provided in the sensitizing emulsion of Freund's adjuvant. Stimulation of lymph node cells from animals receiving HSA in IFA occurred only at an early interval after sensitization, at a time when cutaneous basophil hypersensitivity was readily elicited in the intact animal. The entire cellular response, in culture or in vivo, was transient and could not be detected 6 wk after sensitization. By contrast, sensitization with the same dose of HSA in CFA led to a persistent and evolving cellular response similar to that described in the preceding paper (13). 1 wk after sensitization significant lymphocyte stimulation was demonstrated only at a single high antigen concentration, but by 6 wk stimulation was observed over the entire 6  $\log_{10}$  range of antigen concentration tested. The maximal stimulatory index of 5-fold observed at 6 wk with HSA in CFA was com-

parable to that recorded at 1 wk in the IFA group. However, the absolute level of thymidine incorporation was two to three times higher in the CFA group at this later interval, and there were approximately twice as many lymphoid cells in the enlarged nodes draining the site of a CFA injection. At both 1 and 6 wk animals sensitized with a reduced amount of tubercle bacilli (CFA 1:10) gave an *in vitro* response intermediate between that induced by IFA and CFA immunization.

We were not able to demonstrate significant inhibition of macrophage migration in peritoneal exudates following immunization with HSA in IFA, contrary to earlier reports by Carpenter and Brandriss (15) and by Gerety et al. (16). The success of these groups may reflect the use of the more sensitive explant technique (15) or the different antigens studied. In any case, it is clear from the literature and from our data that much larger amounts of migration-inhibitory factor (MIF) are produced following sensitization with CFA than with IFA and that MIF production is associated with maturity of the cellular immune response (15-17). In our hands, nearly maximal inhibition was obtained with 10  $\mu\text{g}/\text{ml}$  of antigen, and others have reported inhibition with different antigens at concentrations several  $\log_{10}$  dilutions lower (17); this is consistent with the possibility that lymphocytes reacting at low antigen concentration are responsible for MIF production.

In the preceding paper (13) the appearance of lymphocytes reacting at low antigen concentration *in vitro* was associated with a progressive increase in the degree of induration of delayed reactions. Reactivity at high antigen concentration was associated with nonindurated CBH reactions in animals recovering from tolerance. With further recovery, suppressed animals developed indurated skin tests and exhibited lymphocyte reactivity at lower antigen concentration in culture. A similar correlation was observed in the present study. Sensitization with 100  $\mu\text{g}$  HSA in IFA permitted stimulation with no less than 10  $\mu\text{g}$  HSA/ml, while cells from animals sensitized with the same amount of HSA in CFA reacted with as little as 0.1  $\mu\text{g}/\text{ml}$ . These data, then, associate the indurated mononuclear response typical of mature delayed reactions with the presence of lymphocytes capable of proliferation at low antigen concentrations. Conversely, CBH reactions were associated with the appearance of lymphocytes reacting only at high antigen concentration. Appreciation of this difference in lymphocyte reactivity provides at least one explanation for the difficulty encountered in the production of complete tolerance in animals sensitized with IFA or CFA 1:10. Immunization with either of these adjuvants favors the early production of cells reacting at high antigen concentration, precisely those cells least susceptible to a suppressing injection (13). By contrast, CFA elicits only a slight and transient proliferation of such cells, selecting a population reacting at lower antigen concentration in culture that could be more readily suppressed *in vivo* by intravenous antigen.

Studies of antigen clearance and distribution were undertaken to elucidate

the differences observed in response to identical quantities of antigen incorporated in different adjuvants. Sensitization with IFA produced an early pulse of antigen release from the injection site, considerably greater than that observed in animals sensitized with CFA (Fig. 4). Levels in blood and lymphoid tissues 24 hr after 100  $\mu$ g HSA in IFA approximated those seen at a similar interval after intravenous injection of 25–50  $\mu$ g HSA in saline. Systemic dissemination of this amount of antigen could account for the split tolerance produced in guinea pigs after “sensitization” with large quantities of antigen in IFA (18, 19), and might also explain the lack of lymphocyte reactivity at very low antigen concentration observed *in vitro* in our present study after immunization with 100  $\mu$ g HSA in IFA.

Considerably more HSA accumulated in draining lymph nodes when tubercle bacilli were omitted from the sensitizing emulsion. Maximal concentrations were achieved at 1 wk when specific activities were 3–6-fold lower in the CFA sensitized animals, due both to the greater absolute amount of antigen in the IFA nodes and to the greater enlargement of nodes produced by tubercle bacilli. It is tempting to conclude that the higher concentration of antigen accumulating after IFA sensitization favors proliferation of a cell population reactive at high HSA concentration, predicted by the antigen-selection theory of Siskind and Benacerraf (20) and actually observed *in vitro* in our present study.

At later intervals, differences in antigen concentration were even more striking, with specific activities 11 to 13 times greater in the IFA group at 6 wk. A large part of this difference can be attributed to a 5-fold reduction in the absolute amount of antigen present in draining nodes stimulated with CFA. Considering that antigen was cleared from footpads at a similar rate with all adjuvants employed and that the relative distribution of antigen among different organs did not change between 1 and 6 wk, it seems probable that animals sensitized with CFA catabolized antigen at an enhanced rate. Whatever the mechanism involved, the steady decrease in antigen concentration observed in the lymph nodes of CFA-stimulated animals would favor the selection of cells reacting at progressively lower antigen concentration *in vitro* (20), an evolution which, in fact, occurs. Whether the continued presence of high concentrations of antigen in lymph nodes draining IFA depots is related to the early termination of cellular immunity, either as cause or effect, cannot be decided at present.

Conflicting reports have attempted to correlate antigen-specific stimulation of thymidine uptake *in vitro* either with delayed hypersensitivity (21–23) or with antibody production (24–26). In the preceding paper (13) lymphocyte stimulation was observed 1 wk after immunization with 10  $\mu$ g HSA in Freund's complete adjuvant when delayed cutaneous reactivity was well established in the absence of detectable antibody. A similar correlation was observed at 4 wk in sensitized animals recovering from a 5 mg suppressing injection. By con-

trast, antigen-induced lymphocyte stimulation has been reported in the absence of delayed hypersensitivity following the intravenous or intracutaneous administration of soluble or particulate antigens to guinea pigs (25), rabbits (24, 26), and primates (27). However, Turk and Heather (28), Dvorak et al. (10), and Richerson<sup>5</sup> have produced Jones-Mote or cutaneous basophil hypersensitivity administering soluble antigens by these routes. The close correlation of lymphocyte stimulation with CBH reported here is of interest in that CBH is a cell-mediated reaction, passively transferred with lymph node cells, but not with serum. At the same time, CBH is not associated with indurated reactions in vivo or appreciable MIF production in vitro, and the delayed erythematous reactions of CBH may have been ignored or discounted in some studies. While numerous reports have described lymphocyte stimulation in patients with reagenic allergy who do not develop delayed skin reactions, Brostoff and Roitt (23) have recently shown that administration of antihistamine with skin test antigen blocks the immediate response and permits the expression of a weakly indurated reaction that is delayed in onset.

Taken together, these observations suggest that cellular immunity, like antibody formation, encompasses a heterogeneous group of reactions, one of which is classic delayed hypersensitivity. They further suggest that lymphocyte stimulation in animals or patients who do not exhibit classic delayed cutaneous reactivity may nonetheless reflect the presence of other forms of cellular immunity, either overt or latent. Brostoff and Roitt (23) have also suggested that the blast cell produced in vitro and observed in vivo is a thymus-derived intermediate whose progeny mediate cellular sensitivity on the one hand and cooperate in antibody production on the other. This model is appealing in that it would predict the association of lymphocyte stimulation, CBH, and delayed hypersensitivity here reported. However, proliferation of antibody-forming cells and their precursors has been demonstrated (24) and it is not possible to exclude their possible contribution to the thymidine uptake observed in these experiments.

#### SUMMARY

Antigen-mediated stimulation of thymidine incorporation was demonstrated in lymph node cells from guinea pigs immunized with 100  $\mu\text{g}$  human serum albumin in either Freund's incomplete or Freund's complete adjuvant. Animals receiving HSA in IFA exhibited both cutaneous basophil (Jones-Mote) hypersensitivity and lymphocyte stimulation at 1, but not at 6 wk after immunization. Significant stimulation required  $\geq 10 \mu\text{g}$  HSA/ml of culture. Sensitization with HSA in CFA produced delayed hypersensitivity and permitted lymphocyte stimulation at both 1 and 6 wk. Stimulation was observed with as little as 0.1  $\mu\text{g}$  HSA/ml at the later interval.

<sup>5</sup> Dr. H. B. Richerson, personal communication.

Administration of 5 mg HSA intravenously at the time of sensitization with 100  $\mu$ g HSA in IFA reduced but did not eliminate both CBH and lymphocyte stimulation at 1 wk.

Antigen-specific inhibition of macrophage migration could be demonstrated with exudates from animals immunized with HSA in CFA, but not with HSA in IFA at 3 wk after sensitization.

HSA was cleared from depots of CFA and IFA at similar rates, but significantly more antigen appeared in the plasma and subsequently in the draining lymph nodes following administration in IFA. Conversely, accumulated antigen disappeared more rapidly following CFA immunization.

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