THE INDUCTION OF CELL-MEDIATED IMMUNITY AND TOLERANCE WITH PROTEIN ANTIGENS COUPLED TO SYNGENEIC LYMPHOID CELLS*

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One of the oldest paradoxes in immunology is that the kind of immunological response obtained depends critically upon the route of administration of antigen. Nowhere is this better illustrated than in the case of contact sensitivity to DNFB (1-fluoro-2,4-dinitrobenzene). In mice, DNFB applied topically to the skin produces contact sensitivity. The same compound injected intravenously produces profound specific immunologic unresponsiveness (tolerance) to DNFB (1). The understanding and resolution of this paradox requires detailed knowledge of the different methods by which the host processes the topical or i.v. antigens. A large body of data indicates that the real immunogen (for topical DNFB) and the real tolerogen (for i.v. DNFB) are reaction products produced by covalent coupling of DNFB to self components (2). We do not know yet what these products are, but we have extensively studied the tolerance to DNFB induced by treating animals with DNFB coupled to syngeneic lymphoid cell membranes (DNP-LC) (3, 4). This unresponsiveness has been shown to be an extremely efficient, exquisitely specific form of tolerance. In addition, we have separated the mechanisms of tolerance in this system into two distinct pathways (5). Mice injected with DNP-LC may appear tolerant by virtue of one or the other (or both) of two antigen-specific mechanisms: (a) a rapidly induced, long-lasting period of clone inhibition, and (b) a transient period of suppressor T-cell (Tₕ) activity. In addition to the profound tolerogenic activity of hapten-modified lymphoid cells on T-cell-mediated responses, this form of tolerogen has also been shown to be effective for induction of unresponsiveness in B-cell responses (6, 7).

In thinking about the role of membrane-bound DNP in inducing tolerance, we wanted to know whether these concepts could be generalized to apply to protein antigens. To study this in mice, one needs convenient methods to induce cell-mediated immunity (CMI) to proteins and to test the immunogenicity and tolerogenicity of membrane-bound proteins.

Recently, improved methods of inducing and eliciting CMI to proteins in mice have been developed, using complete Freund's adjuvant (CFA) for induction (8) and measurement of ear swelling for elicitation (9). Other work has shown that contact hypersensitivity to simple chemicals can be induced by injection of hapten-coupled autologous cells (10, 11). More recent experiments indicate that vastly different responses can be elicited with hapten-modified cells depending on the route of injection (12, 13). i.v. injection of spleen cells modified with trinitrophenyl (TNP) or azobenzenearsenonate lead to the induction of both tolerance and Tₕ, whereas s.c. injection of hapten-modified cells lead to the induction of a delayed-type contact sensitivity response.

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Abbreviations used in this paper: ABA, azobenzenearsenonate; BSS, balanced salt solution; C', guinea pig complement; CFA, complete Freund's adjuvant; CMI, cell-mediated immunity; Cyt C, horse cytochrome C; DNFB, 1-fluoro-2,4-dinitrobenzene; DTH, delayed type hypersensitivity; ECDI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl; HoGG, horse gamma globulin; HoGG-LC, HoGG-modified lymphoid cells; MHC, major histocompatibility complex; NRS, normal rabbit serum; OVA, ovalbumin; Tₕ, delayed hypersensitivity T cells; Tₚ, suppressor T cells; TNP, trinitrophenyl.
Therefore, we followed the earlier experiments of Battisto and Bloom (14, 15) who showed that bovine gamma globulin coupled to isologous guinea pig spleen cells could induce tolerance to both humoral antibody production and delayed hypersensitivity responses, and combined those concepts with the recent developments concerning CMI in the mouse.

The present study was designed (a) to produce a reliable method for inducing CMI to protein antigens in a mouse model without using CFA, and (b) to examine the immunogenicity and tolerogenicity of membrane-bound proteins in this model. Furthermore, we compared the tolerogenicity of free and membrane-bound protein, and explored the dual mechanisms involved in this tolerance.

Materials and Methods

Animals. Female BALB/c mice were obtained from Cumberland Farms, Clinton, Tenn. Male CBA and SJL mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. Mice were age-matched for each experiment and received pelleted food and water ad lib.

Antigens and Chemicals. Cohn Fraction II of horse gamma globulin (HoGG) (lot 82-254) and crystallized ovalbumin (OVA) (lot 95-051-2) were both obtained from Miles Laboratories Inc., Miles Research Products, Elkhart, Ind. Cytochrome C (Cyt C) from horse heart (type III—Lot 1BC-7540) was obtained from Sigma Chemical Co., St. Louis, Mo. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (ECDI) was obtained from Story Chemical Corp., Muskegon, Mich.

Preparation of Antigen-Modified Lymphoid Cells. Spleen cell suspensions were prepared in Hanks’ balanced salt solution (HBSS) as described previously (4). Erythrocytes were lysed by treatment with isotonic Tris-buffered ammonium chloride, the cells washed two times with HBSS, and once with 0.9% NaCl. 400 × 10^8 cells were pelleted in a 17 × 100 mm Falcon plastic tube and resuspended in 1.5 ml of antigen diluted in 0.9% NaCl (40 mg/ml for HoGG and 20 mg/ml for Cyt C). To the above, 0.25 ml of freshly prepared ECDI (100 mg/ml in saline) was added. The reaction was allowed to proceed for 1 h at 4°C, after which the antigen-modified cells were washed three times with HBSS, and adjusted to a concentration of 10^7/ml. To determine the amount of HoGG coupled, 125I-labeled HoGG was added to the reaction mixture in tracer amounts. A series of five separate experiments showed that an average of 5.33 ± 0.45 µg of HoGG was coupled per 10^7 lymphoid cells.

Deaggregation of HoGG. 30 mg/ml HoGG was deaggregated by centrifugation at 40,000 rpm for 150 min in a swinging bucket SW 50.1 rotor (16). The upper third of the solution was removed and diluted to a concentration of 50 µg/ml.

Sensitization and Elicitation of CMI. Sensitization to various proteins was accomplished by two methods. First, antigens in the optimal concentrations were emulsified in CFA (Difco Laboratories, Detroit, Mich.). Mice were injected s.c. at the base of the tail with a total emulsion vol of 50 µl (17). Optimal sensitization amounts for the various antigens were as follows: HoGG—250 µg; OVA—100 µg; and Cyt C—100 µg. In the second method, antigen-modified cells were injected s.c. (12). Mice were injected in four positions beneath the dorsal skin with a total of 3.5 × 10^7 HoGG-LC in a total vol of 0.2 ml. For elicitation of CMI, mice were ear challenged with the appropriate antigen 7 d after antigen-CFA or 6 d after antigen-modified lymphoid cells (LC). Ear thickness was quantitated using a Mitutoya engineer’s micrometer (Schlesinger’s Tools, Brooklyn, N. Y.); the dorsal surface of the ear was then injected s.c. with 10 µg of antigen contained in 10 µl of saline using a 27 gauge needle. 24 h postantigen challenge, the degree of ear thickness was again measured and results expressed in units of 10^-4 inches.

Induction and Transfer of Sensitivity. For transfer of sensitivity, mice were sensitized by s.c. injection of 60 × 10^6 HoGG-LC divided into four sites on the dorsal skin and the two front footpads. 4 d postsensitization, draining lymph nodes (inguinal, axillary, and brachial) were collected and 10^6 viable cells transferred to normal, syngeneic recipients. Recipient mice were ear challenged within 1 h of cell transfer and the degree of ear swelling measured 24 h later.

Induction and Transfer of Tolerance. Mice were injected i.v. with 10^9 HoGG-LC or 10^8 Cyt C-LC at varying times before sensitization or transfer of tolerance. For transfer of tolerance, peripheral and mesenteric lymph nodes and/or spleens were collected and single cell suspensions prepared in Mishell-Dutton balanced salt solution (BSS). From 10^9 to 1.6 × 10^9 donor...
lymphocytes were injected i.v. into normal recipients. Control mice received either no cells or an equivalent number of cells from normal donors. The recipient and control groups were sensitized within 1 h after transfer and challenged 6–7 d later. The degree of donor tolerance or tolerance transferred to normal recipients was expressed as percent tolerance according to the following formula using ear swelling values:

\[
\text{percent tolerance} = 1 - \frac{\text{experimental} - \text{negative control}}{\text{positive control} - \text{negative control}} \times 100%.
\]

**Anti-θ Serum Treatment.** Rabbit anti-brain-associated θ serum was prepared and absorbed according to the method of Golub (18). Lymphoid cells were reacted with 1.0 ml of a 1:10 dilution of normal rabbit serum (NRS) or anti-θ serum/10⁶ cells for 45 min at 4°C, washed once in BSS, and then incubated with 1.0 ml of a 1:6 dilution of guinea pig complement (containing 10 μg/ml DNAse)/10⁶ cells for 30 min at 37°C. The cells were then washed twice in BSS and recounted for cell transfer.

**In Vitro Culture.** After determination of in vivo ear swelling, draining lymph nodes were used as a source of cells for antigen-induced proliferation. For mice sensitized in the base of the tail, these consisted of inguinal and periaortic nodes. For mice sensitized s.c. with HoGG-LC, these consisted of inguinal, axillary, and brachial nodes. Cell suspensions were prepared and washed in BSS and the cells were cultured in tissue culture medium prepared exactly as described by Corradin et al. (17). Briefly, this consisted of modified Click's (19) medium containing 10% fetal calf serum and 2-mercaptoethanol. Cell suspensions were dispensed into Costar flat-bottom microwell plates, each well receiving 0.2-ml vol containing 4 × 10⁶ lymphoid cells. Antigens (100 μg/well of HoGG, OVA, or Cyt C) were added in a 10 μl vol. The plates were incubated at 37°C in a humidified atmosphere of 5% CO₂, 95% air for 120 h. Cultures were pulsed for the final 24 h of culture with 1 μCi (in 10 μl) of [³H]thymidine (6 Ci/m mol, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.). Cultures were harvested with an automated sample harvester, and radioactive measurements of trichloroacetic acid insoluble material determined in a Packard liquid scintillation counter.

**Statistical Analyses.** The statistical significance of differences in ear swelling reactions between experimental groups was calculated with the Student's t test.

**Results**

*Induction of Sensitization with Antigen-CFA or Antigen-Modified Lymphoid Cells.* In early attempts to produce a reliable method of inducing CMI to protein antigens, we compared two regimens: the sensitization of BALB/c mice with HoGG-CFA in the base of the tail vs. the s.c. injection of HoGG-LC into the back. Table I shows a comparison of these two methods in terms of both ear swelling in vivo and antigen-induced T-cell proliferation in vitro. Both methods resulted in readily detectable ear swelling and in vitro proliferative responses. Peak responsiveness with HoGG-CFA occurred with 250 μg of antigen (exp. 1—group C). Peak responsiveness upon s.c. sensitization with HoGG-LC occurred with 3–5 × 10⁷ modified cells (exp. 2—groups B and C). The degree of in vivo ear swelling correlated closely with the extent of in vitro proliferation.

The kinetics of the ear swelling reaction postchallenge had typical delayed characteristics (data not shown). There was very little response at 4 h. Extensive swelling was present at 24 h; it declined dramatically after that, but was still significant at 48 h.

To determine the phenotype of the cell responsible for the observed reactivity, 10⁸ viable lymph node cells from sensitized mice were treated in vitro with either NRS + C' or anti-θ + C' and transferred to normal, syngeneic recipients. A third group received 0.4 ml of serum from sensitized animals. The results shown in Fig. 1 demonstrate that a small, but significant, degree of transfer can be accomplished with
| Exp. no. | Exp. group | Sensitization regimen | Route* | Ear swelling §  | In vitro response $ | Δ cpm |
|---------|------------|-----------------------|--------|----------------|------------------|-------|
|         |            |                       |        |                |                  |       |
| 1       | A          | 50 μg HoGG-CFA i.t.   | 22.5 ± 1.3 | 19,070         |                  |       |
|         | B          | 100 μg HoGG-CFA i.t.  | 23.9 ± 3.5 | 16,600         |                  |       |
|         | C          | 250 μg HoGG-CFA i.t.  | 38.5 ± 4.9 | 26,580         |                  |       |
|         | D          | 500 μg HoGG-CFA i.t.  | 20.6 ± 2.0 | 29,030         |                  |       |
|         | E          | Saline-CFA i.t.       | 1.9 ± 2.2  | -1,800         |                  |       |
| 2       | A          | 10^7 HoGG-LC s.c.     | 30.1 ± 2.4 | 16,830         |                  |       |
|         | B          | 3 × 10^7 HoGG-LC s.c. | 46.3 ± 3.4 | 28,570         |                  |       |
|         | C          | 5 × 10^7 HoGG-LC s.c. | 51.7 ± 3.1 | 29,190         |                  |       |
|         | D          | 5 × 10^7 Sham-LC s.c. | 5.5 ± 1.1  | -1,800         |                  |       |

* Route of sensitization: i.t. = intratail; s.c. = subcutaneously in the back.
§ Mean ear swelling response of groups of four to five mice ± SEM.
$ Mean Δ cpm in vitro response (X cpm 100 μg/culture HoGG - X cpm Nil) for quadruplicate 5-d cultures.

Fig. 1. Transfer of sensitivity induced by subcutaneous injection of HoGG-modified lymphoid cells is mediated by θ-sensitive T cells, not by immune serum. Donor mice were sensitized by s.c. injection with a total of 6 × 10^7 HoGG-LC 4 d before transfer of draining LN cells. Values represent mean 24 h ear swelling ± SEM for groups of five mice. NS, not significantly different than negative control (group D).

sensitized LN cells (group A). However, treatment of the cells with anti-θ + C' before transfer abrogated this reaction. Recipients receiving serum from sensitized donors also showed no significant ear swelling. Together, the data from the kinetic and transfer experiments indicate that the response is delayed-in-time and requires T cells.

Comparison of the Tolerogenic Nature of Equivalent Amounts of Free and Cell-Bound Antigen. In recent years, our laboratory has been extensively studying the concept that T cells are best tolerized by membrane-associated antigens. In this case, we used the model of contact sensitivity to DNFB and studied the induction of tolerance with DNP-LC (4, 5). We asked if protein tolerogens showed the same phenomenon. To do this, we compared the tolerogenicity of free HoGG compared with HoGG coupled to lymphoid cells with and without carbodimide. A series of experiments using 125I-labeled HoGG revealed that 5.3 ± 0.45 μg of HoGG was coupled per 10^7 lymphoid cells by the carbodimide procedure and 1.28 ± 0.22 μg/10^7 cells without carbodimide. Therefore,
### Table II
Comparison of the Tolerogenic Capacity of Soluble vs. Cell-Bound HoGG in BALB/c Mice

| Exp. no. | Exp. group | Day -7 i.v. tolerogen* | Day 0 sensitization‡ | In vivo response§ | In vitro response¶ |
|---------|------------|-----------------------|----------------------|------------------|------------------|
|         |            |                       |                      | Ear swelling | Tolerance |
|         |            |                       |                      | × 10^{-4} inch | SEM | Δ cpm |
| 1       | A 10^6 HoGG-LC (50 µg bound) (+ ECDI) | 250 µg HoGG-CFA | 12.9 ± 0.9 | 84.5 | 1,130 |
|         | B 10^6 HoGG-LC (13 µg bound) (- ECDI) | 250 µg HoGG-CFA | 29.5 ± 3.2 | 45.7 | 23,950 |
|         | C 50 µg Deaggregated HoGG | 250 µg HoGG-CFA | 49.4 ± 2.6 | -0.7 | 48,240 |
|         | D 50 µg Native HoGG | 250 µg HoGG-CFA | 44.4 ± 2.5 | 11.0 | 38,490 |
|         | E 0.5 mg Native HoGG | 250 µg HoGG-CFA | 45.2 ± 1.3 | 8.9 | 42,290 |
|         | F 5.0 mg Native HoGG | 250 µg HoGG-CFA | 43.9 ± 2.6 | 11.9 | 45,810 |
|         | G -- | 250 µg HoGG-CFA | 49.1 ± 2.7 | -- | 43,210 |
|         | H -- | Saline-CFA | 6.3 ± 2.2 | -- | -5,890 |
| 2       | A 10^6 HoGG-LC (50 µg bound) | 3 × 10^7 HoGG-LC s.c. | 7.9 ± 1.3 | 87.5 | -1,200 |
|         | B -- | 3 × 10^7 HoGG-LC s.c. | 37.4 ± 1.5 | -- | 14,720 |
|         | C -- | -- | 3.7 ± 0.8 | -- | -930 |

* Mice were tolerized by i.v. injection of the indicated materials 7 d before sensitization.
‡ Mice in exp. 1 were sensitized with 250 µg HoGG-CFA i.t.; mice in exp. 2 were sensitized 3 × 10^7 HoGG-LC s.c.
§ Mean ear swelling response for groups of four mice ± SEM at 7 d (exp. 1) or 6 d (exp. 2) postsensitization.
¶ Mean Δ cpm in vitro responses (X cpm 100 µg/culture HoGG - X cpm Nil) for quadruplicate 5-d cultures.
† Significantly less than positive controls P < 0.001.

BALB/c mice were injected i.v. with either 10^6 HoGG-LC or the equivalent amount of native HoGG or deaggregated HoGG on day-7 (Table II). Additional groups received 10 × (0.5 mg) or 100 × (5.0 mg) of native HoGG. On day 0, experimental and positive control mice in exp. 1 received 250 µg HoGG-CFA i.t. and in exp. 2 received 3 × 10^7 HoGG-LC s.c. Negative controls received saline-CFA or sham-modified cells, respectively. All groups were ear challenged with 10 µg, and ear swelling and in vitro proliferation determined 24 h later. The results shown in Table II indicate that prior injection of soluble HoGG (either native or deaggregated) up to a dose of 5.0 mg had no effect on the CMI response to HoGG-CFA as measured via ear swelling or in vitro proliferation. However, i.v. injection of 10^6 HoGG-LC profoundly suppressed the development of both the in vivo and in vitro manifestations of the CMI response induced by antigen-CFA (exp. 1—group A) or by antigen-modified lymphoid cells given s.c. (exp. 2—group A). I.v. injection of LC, which had been incubated with HoGG but without ECDI, led to intermediate levels of unresponsiveness measured both in vivo and in vitro (exp. 1—group B). The degree of tolerance is approximately what would be expected with 1/4 the dose of ECDI-coupled HoGG (see Fig. 2 and later discussion of dose-response). Suppression of the CMI response to HoGG by prior i.v. injection of HoGG-LC was not restricted to the BALB/c strain, as CBA mice were also tolerized by this treatment (data not shown).
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Ear Swelling ($x 10^{-4}$ inch) ± SEM

| Group | Day of Tolerization | Dose of HoGG-LC i.v. | Ear Swelling ($x 10^{-4}$ inch) ± SEM | In Vitro b
|-------|---------------------|----------------------|--------------------------------------|---------------------
| A     | -                   | (Pos Con.)           |                                     | 28.410
| B     | -7                  | $10^7$               |                                     | 10.730
| C     | -7                  | $5 \times 10^7$      |                                     | 7.060
| D     | -7                  | $10^8$               |                                     | 7.910
| E     | -4                  | $10^8$               |                                     | 100
| F     | -1                  | $10^8$               |                                     | 7.0
| G     | -                   | (Neg Con.)           |                                     | -2.710

Fig. 2. Dose-response and time-course of tolerance induction using i.v. injection of HoGG-modified lymphoid cells. Bars represent mean ear swelling ± SEM for groups of four mice. Mean Δ cpm in vitro response (X cpm 100 μg/culture HoGG—X cpm Nil) for quadruplicate 5-d cultures. Values in parentheses represent the percentages of suppression. * Significantly lower than positive controls P < 0.01.

TABLE III
Specificity of Tolerance Induced by i.v. Injection of HoGG Modified Syngeneic LC (HoGG-LC) in BALB/c Mice

| Exp. group | Day -7 tolerogen | Day 0 sensitization | Day +7 challenge | In vivo response\* | In vitro response\* | Ear swelling (% \* SEM) | Tolerance vs. \* HoGG vs. OVA | Δ cpm |
|------------|------------------|---------------------|------------------|-------------------|-------------------|-------------------------|--------------------------------|-------|
| A          | $10^8$ HoGG-LC i.v. | 250 μg HoGG-CFA i.t. | HoGG             | 11.5 ± 3.6        | 88.28             | 140                     | -15                            |
| B          | —                | 250 μg HoGG-CFA i.t. | HoGG             | 62.1 ± 2.1        | —                 | 53,420                  | -210                           |
| C          | $10^8$ HoGG-LC i.v. | 100 μg OVA-CFA i.t. | OVA              | 66.4 ± 2.5        | -3.2              | -1,560                  | 21,450                         |
| D          | —                | 100 μg OVA-CFA i.t. | OVA              | 64.5 ± 3.0        | —                 | -2,490                  | 20,300                         |
| E          | —                | Saline-CFA i.t.     | HoGG             | 4.7 ± 1.5         | —                 | -1,430                  | 330                            |
| F          | —                | —                   | OVA              | 4.7 ± 0.9         | —                 | -                        |                                 |

\* Mean ear swelling response for groups of four to five mice ± SEM.

\# Mean Δ cpm in vitro response of draining LN cells vs. either 100 μg HoGG or 100 μg OVA per culture for quadruplicate 5-d cultures.

§ Significantly less than positive controls P < 0.001.

Specificity of Tolerance Induction by Antigen-Modified Lymphoid Cells. We were next concerned with the specificity of the tolerance induced by antigen-modified LC. To answer this question, two groups of mice were tolerized on day -7 with $10^8$ HoGG-LC; and on day 0, one group was sensitized with HoGG-CFA and the other with OVA-CFA, along with the appropriate positive and negative control groups. All mice were ear challenged with 10 μg of the appropriate antigen on day 7, and increased ear swelling and in vitro proliferation determined 24 h later. As can be seen in Table III,
immunity and tolerance with antigen-modified lymphocytes

**Table IV**
Specificity of Tolerance Induced by i.v. Injection of Cytochrome C Modified Syngeneic LC (Cyt C-LC) in SJL Mice

| Exp. group | Day -7 tolerogen | Day 0 sensitization | Day +7 challenge | In vivo response* | In vitro response‡ |
|------------|------------------|---------------------|------------------|------------------|-------------------|
|            |                  |                     |                  | Ear swelling     | Tolerance vs. Cyt C vs. HoGG | \( \times 10^{-4} \) inch | % | \( \Delta \) cpm |
| A          | \( 10^6 \) Cyt C-LC | 100 µg Cyt C-CFA i.t. | Cyt C           | 6.4 ± 1.1        | 94.8§               | 4,600          | 250  |
| B          |                  | 100 µg Cyt C-CFA i.t. | Cyt C           | 30.2 ± 1.8       | —                  | 15,610        | —   | —1,140 |
| C          | \( 10^7 \) Cyt C-LC | 250 µg HoGG-CFA i.t. | HoGG            | 36.5 ± 2.9       | 8.7                | —200          | 131,870 |
| D          |                  | 250 µg HoGG-CFA i.t. | HoGG            | 39.5 ± 5.7       | —                  | 560           | 103,740 |
| E          |                  | Saline-CFA i.t.     | Cyt C           | 4.7 ± 1.5        | 560                | 103,740       | 50  | —290   |

* Mean ear swelling response for groups of four to five mice ± SEM.
‡ Mean \( \Delta \) cpm in vitro response of draining LN cells vs. either 100 µg Cyt C or 100 µg HoGG per culture for quadruplicate 5-d cultures.
§ Significantly less than positive controls \( P < 0.001 \).

Mice tolerized with HoGG-LC and sensitized with HoGG-CFA (group A) were nearly completely tolerant as measured by ear swelling or in vitro cell proliferation when compared to positive controls (group B). However, similarly treated mice which were sensitized with OVA-CFA (group C) reacted the same as the OVA-positive controls (group D). Thus, the tolerant state induced by i.v. injection of HoGG-LC appears to be antigen-specific.

Table IV illustrates two additional points in this regard. The experimental design is similar to the previous experiment, except that SJL mice were tolerized with horse Cyt C-modified LC, and sensitized with either Cyt C-CFA or HoGG-CFA. Again, tolerance was specific as measured both in vivo or in vitro. Mice were unresponsive to Cyt C, but responded normally to HoGG. This experiment shows that SJL mice are appropriate for these studies and that the response to a very different protein (Cyt C) can be suppressed by i.v. injection of antigen-coupled lymphoid cells.

**Dose-Response and Time-Course of Tolerance Induction with Antigen-Modified Lymphoid Cells.** We next asked about the optimal quantities of HoGG-LC needed to induce tolerance and how long before sensitization do they need to be given? Groups of BALB/c mice were injected i.v. with \( 10^7 \), \( 5 \times 10^7 \), or \( 10^8 \) HoGG-LC 7 d before sensitization, and with \( 10^8 \) HoGG-LC on day 4 and day 1 before sensitization with 250 µg HoGG-CFA i.t. The results are shown in Fig. 2 and illustrate two points. First, the injection of \( 10^8 \) HoGG-LC at 7, 4, or 1 d before sensitization leads to levels of tolerance which are approximately the same (groups D–F). This result suggests that induction of unresponsiveness occurs very rapidly in that mice tolerized 1 d before sensitization respond as poorly as mice tolerized 4 or 7 d before sensitization. Second, increasing the amount of antigen-modified LC given 7 d before sensitization leads to increased levels of unresponsiveness suggesting that its induction is dose-dependent (compare groups B–D). The in vitro response of mice given \( 10^8 \) HoGG-LC at days...
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**Table V**

*Kinetics of Tolerance Induction vs. the Development of Suppressor Cells*

| Group | Tolerogen day* | Cells transferred on day 0 | Day 0 sensitization | In vivo response‡ | In vitro§ |
|-------|---------------|-----------------------------|----------------------|-------------------|-----------|
|       |               |                             | × 10−4 inch ± SEM    | Ear swelling      | Tolerance | A cpm     |
| A     | 10⁷ HoGG-LC   | None—tolerance control      | 3 × 10⁷ HoGG-LC s.c. | 10.7 ± 0.6        | 76.5      | -2,010    |
| B     | 10⁷ HoGG-LC   | 8 × 10⁷ LN + 8 × 10⁷ SP from group A | 3 × 10⁷ HoGG-LC s.c. | 18.7 ± 1.6        | 55.9      | 8,220     |
| C     | 10⁷ HoGG-LC   | None—tolerance control      | 3 × 10⁷ HoGG-LC s.c. | 11.8 ± 2.8        | 73.7      | -2,820    |
| D     | 10⁷ HoGG-LC   | 8 × 10⁷ LN + 8 × 10⁷ SP from group C | 3 × 10⁷ HoGG-LC s.c. | 22.2 ± 1.8        | 46.9      | 6,300     |
| E     | 10⁷ HoGG-LC   | None—tolerance control      | 3 × 10⁷ HoGG-LC s.c. | 8.6 ± 1.5         | 82.0      | -2,310    |
| F     | 10⁷ HoGG-LC   | 8 × 10⁷ LN + 8 × 10⁷ SP from group E | 3 × 10⁷ HoGG-LC s.c. | 38.0 ± 1.3        | 6.2       | 7,660     |
| G     | —             | None—positive control       | 3 × 10⁷ HoGG-LC s.c. | 40.4 ± 3.6        | —         | 7,430     |
| H     | —             | None—negative control       | 3 × 10⁷ Sham-LC s.c. | 1.6 ± 0.6         | —         | -830      |

* BALB/c mice were tolerized with 10⁷ HoGG-LC on days -7, -4, and -1. On day 0, half of each group was sensitized with 3 × 10⁷ HoGG-LC s.c. and served as tolerant controls. The other half of each group served as donors of tolerant LN and spleen cells which were transferred to normal recipients who were sensitized with 3 × 10⁷ HoGG-LC within 1 h of cell transfer.

‡ Values represent mean ear swelling responses for groups of four mice ± SEM.

§ Mean A cpm (X 100 µg/culture HoGG - X cpm Nil) for quadruplicate 5-d cultures.

Values significantly lower than positive controls P < 0.01.

-4 or -1 is completely suppressed (groups E and F) while mice receiving the same dose of HoGG-LC at -7 d shows a small degree of in vitro proliferation (group D).

Unresponsiveness Induced by Antigen-Modified Lymphoid Cells is Transferable by Antigen-Specific Suppressor T Cells. We next asked if the unresponsive state induced by prior i.v. injection of HoGG-LC was transferable to normal recipients (Table V). Groups of eight BALB/c mice were injected with 10⁷ HoGG-LC i.v. 7, 4, or 1 d before transfer. On day 0, four of the mice in each group were sensitized by s.c. injection of 3 × 10⁷ HoGG-LC in the back; these served as donor tolerant controls. The other four mice in each group were sacrificed and 8 × 10⁷ spleen plus 8 × 10⁷ LN cells transferred to normal recipients, which were also sensitized with 3 × 10⁷ HoGG-LC s.c. Positive controls received the same number of normal cells before sensitization. Negative controls were sensitized with 3 × 10⁷ sham-modified LC. The results shown in Table V indicate that mice given HoGG-LC from 1 to 7 d before sensitization are very unresponsive as measured by in vivo ear swelling or in vitro antigen-induced proliferation (groups A, C, and E). In contrast, 1 d after tolerization with HoGG-LC, mice although fully tolerant exhibited no demonstrable suppressor cells (group F). However, 4 to 7 d after HoGG-LC injection, mice were both fully tolerant and contained cells capable of transferring unresponsiveness to normal recipients (groups B and D). These results suggest that, as in the DNFB system (5), there may be two
The cellular nature and antigen-specificity of the suppressor cells in this system were next examined (Fig. 3). BALB/c mice were tolerized on day -7 with \(10^8\) HoGG-LC. On day 0, LN cells were removed, treated with NRS + C' or with anti-\(\theta + C'\), and transferred into normal recipients. The recipients were sensitized with either HoGG-CFA or OVA-CFA, along with the appropriate control groups. 7 d after sensitization, mice were ear challenged and ear swelling and in vitro proliferation measured 24 h later. Group A shows that the donors of the tolerant LN cells were 88% tolerant and their cells failed to respond in vitro. Recipients of NRS + C'-treated tolerant LN cells (group C) were 46% suppressed, but recipients of anti-\(\theta + C'\)-treated tolerant LN cells (group D) were only 6% suppressed. Thus, the suppression requires cells of the T-cell lineage. The antigen-specificity of the Tc is shown by the fact that recipients of NRS + C'-treated tolerant LN cells who were sensitized and challenged with OVA responded normally both by ear swelling and in vitro proliferation (group G). It should be noted that cells of the suppressed recipients of tolerant LN cells (group C) again responded normally in the proliferation assay.

**Discussion**

This report has examined the development of a CMI response to protein antigens...
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in mice and has described the induction of tolerance in this system. A CMI response to HoGG, OVA, or Cyt C could be induced by two methods: injection of protein emulsified in CFA into the base of the tail or by the s.c. injection into the dorsal skin of protein-coupled syngeneic lymphoid cells. After these methods of sensitization, ear challenge with the appropriate free antigen was followed by ear swelling which was significant at 24 but not at 4 h, typical of a delayed hypersensitivity response. The T-cell dependence of the response is illustrated by the fact that transfer of sensitivity is accomplished by θ-sensitive lymphoid cells, and not by serum. After these sensitization regimens, mice have T cells in the regional nodes which proliferate in vitro when challenged with the appropriate antigen (17).

The use of cell-coupled haptens for the induction of T-cell-mediated contact sensitivity reactions has been described both for guinea pig and mouse models. Baumgarten and Geczy (10) and Polak and Macher (11) showed that DNP-modified lymphocytes injected either i.p. or intradermally in guinea pigs could induce DNP-specific contact sensitivity. More recently, Green et al. and Bach et al. applied this methodology to a mouse model and showed that s.c. injection of TNP- or azobenzene-arsonate (ABA)-modified lymphocytes lead to the development of significant delayed hypersensitivity responses (12, 13). The present study extends this methodology to induction of CMI responses to soluble protein antigens coupled to syngeneic lymphocytes. This induction of CMI responses by s.c. injection of antigen-modified lymphoid cells provides a powerful tool for the study of induction of, genetic constraints on, and mechanisms of, antigen-specific T cells without concern for nonspecific effects of CFA.

To produce tolerance to the CMI induced with protein antigens, the same preparations of protein-modified LC were merely injected i.v. before sensitization. The tolerance produced was (a) rapid, (b) specific, and (c) more efficient (in terms of dose of tolerogen) than that induced by i.v. injection of free protein. Furthermore, i.v. tolerogens also led to the production of antigen-specific T suppressors (Ts) able to transfer suppression to naive recipients. Finally, analysis of the kinetics of the induction of tolerance and of suppression leads us to conclude that in this system, as in tolerance to DNP-LC, two mechanisms of unresponsiveness exist: a rapidly-induced state of clone inhibition (not associated with Ts) and the later development of Ts. 1 d after tolerization with i.v. protein-LC, mice were unresponsive but could not transfer unresponsiveness to normal recipients (i.e. had no demonstrable Ts). 4 and 7 d after tolerogen, mice were unresponsive and also contained Ts. This dichotomy between unresponsiveness due to clone inhibition and that due to Ts is also seen in tolerance to antibody formation induced by deaggregated γ-globulins (20-22) where active suppression is present only briefly, while the tolerant cells remain unresponsive for a much longer period of time.

An interesting feature of the Ts induced by injection of HoGG-LC is that they suppress the TDH cells responsible for mediating the ear swelling response, but fail to suppress the development of the T cell which proliferates in vitro upon antigenic restimulation. We propose two possible explanations for this phenomenon. First, it is possible that Ts act by suppressing the efferent (or elicitation) phase of the CMI response (23). They therefore may not block the development of the in vitro proliferative cells which seems to be a process which occurs during the afferent (sensitization) phase. A second possible explanation is that the Ts may act only on the TDH cell, but not on the in vitro proliferating T cell. It is now clear that in the DNFB contact sensitivity system TDH and proliferating T cells belong to separate T-cell lineages.
Moorhead (24) has shown that TDH cells in that system are nonadherent to nylon wool and are Ia-, while the in vitro proliferating T cell is adherent and Ia+. These findings agree with those of Vadas et al. (25) who showed that in CMI to fowl γ-globulin, the cells responsible for the in vivo transfer of CMI were also Ia-. Experiments to distinguish between these two possibilities and at what level Tₘ mediate in vivo suppression (i.e. induction or expression) are currently being performed.

Mode of Antigen Presentation in T-Cell Function. In analyzing these results, we think in terms of three events and two types of antigen-specific T cells. These are: (a) the development of CMI via stimulation of TDH, (b) rapid induction of tolerance via clone inhibition of these TDH, and (c) slower development of infectious tolerance via stimulation of Tₘ. Thus there are two net results for the TDH—a turn-on (sensitization) or a turn-off (clone inhibition unresponsiveness). In the context of this paper, there is only one result of triggering precursors of Tₘ which is to cause the development of active Tₘ. (Such cells can be non-specifically eliminated, e.g. by cyclophosphamide [5], but we are not aware of specific turn-off signals for Tₘ.)

As a result of an enormous amount of recent research, it is now clear that T cells recognize antigens in association with MHC products (26, 27). Recent studies on the development of contact sensitivity responses in vivo (12) and the induction of T-cell proliferation in vitro (28) have also suggested an important role for macrophage presentation.

Thus, effective immunogenicity seems to involve macrophage-associated antigenic determinants presented to the appropriate T cell in conjunction with MHC products. The elegant transfer experiments of Vadas et al. (27) in CMI to fowl γ-globulin suggested that T cells mediating DTH responses recognize antigen in association with Ia region determinants. It is likely that the antigen-presenting cell in contact sensitivity is an Ia+ macrophage-like Langerhans cell in the skin (29) which thus conjugates with topically applied DNFB. We believe that this is what is recognized by the TDH and what activates it during contact sensitization.

It is difficult to induce CMI to proteins in mice without adjuvants such as CFA. While the action(s) of adjuvants are still not fully understood, it is likely that the intense inflammation may facilitate interaction of macrophages with the suspended protein, thus favoring immunogenic presentation to T cells. In the experiments described here, we found that proteins were immunogenic in CFA but also equally immunogenic if bound to lymphoid cells and given subcutaneously without CFA. We believe that the effectiveness of this regimen depends on the fact that subcutaneous cell-bound antigen is particularly efficient in becoming associated with MHC products of macrophages which are in, or which reach, the regional lymph nodes. It is this macrophage-antigen-major histocompatibility complex (MHC) product complex which is seen by the T cells in the lymph node and which activates them.

In terms of turning off T₈ in the rapid development of unresponsiveness (which we call clone inhibition), it is also clear that the most effective tolerogens are those bound to cells. In contact sensitivity to DNFB, chemicals which don't couple covalently to self are not tolerogenic (1). Furthermore, DNP coupled to proteins or polypeptides may be good tolerogens for B cells (30, 31), but these soluble antigens do not produce rapid clone inhibition in contact sensitivity (4, 32). There is some information that the MHC must be involved in this clone inhibition since DNP-mouse RBC (lacking Ia and poor in H-2) is very inefficient in producing rapid clone
inhibition (4). Thus, again, it is not surprising that protein coupled to nucleated cells containing MHC products can, when presented by the proper route (i.v. not s.c.), induce clone inhibition. This clone inhibition probably occurs after the arrival of blood-borne cell-bound protein in the lymph nodes, as these are the site of immunization by cell-bound protein given s.c. To prevent this immunization, we believe that the i.v. cell-bound protein is delivered to the lymph nodes in a way which allows direct presentation to the antigen receptors on T_{DH}. Frei et al. put forward the concept that immunogenicity involves macrophage processing of antigen, and tolerance involves bypassing the macrophages with direct presentation of antigen to lymphocytes (33). As we presently believe that T cells see antigens best in the context of membrane presentation, this concept is quite compatible with the data showing the tolerogenicity of cell-bound antigens. In this case, therefore, the turn-off signal to the T_{DH} would involve presentation of antigen on a cell membrane, but via a nonimmunogenic (perhaps a nonmacrophage) pathway. Although we do not know what this nonimmunogenic pathway may be, it could involve presentation via nonmacrophage membranes in a situation where the ordinary positive triggering signal is not delivered to the T_{DH}.

It should also be noted that HoGG associated with LN without ECDI is a potent tolerogen (Table II—group 1B). Presumably, the HoGG is bound to LC via Fc receptors. The tolerogenicity of such a cell-protein complex suggests that cell-bound molecules do not have to be covalently linked to the cell membrane.

The third event in these experiments, i.e. the development of T_{s}, also requires the presentation of cell-bound antigen. This appears to be true because, using the arguments in the previous paragraph, materials which are not cell-associated not only fail to produce clone inhibition but also do not produce T_{s}. We have previously shown in suppression of DNFB contact sensitivity (34) that compatibility at the H-2D region of the MHC between the DNP-modified lymphoid membrane tolerogen and the T_{s} donor mouse is both sufficient and necessary for T_{s} induction. Thus, it would appear that in that system the T_{s} precursors are triggered by hapten-modified H-2D region determinants. Whether this relationship is operating in T_{s} induction by protein antigen-modified lymphoid cells is currently under investigation. Recent work in our laboratory has also suggested that I region compatibility between the DNP-LC tolerogen and tolerized mice is necessary for the induction of rapid clone inhibition (i.e. tolerance induced within 1 d). This would explain the fact that i.v. injection of DNP-RBC (lacking I region determinants), as opposed to DNP-LC, does not lead to rapid tolerance induction (4), but does eventually lead to T_{s} induction (3).

Again, however, the production of T_{s} by cell-bound antigen also depends greatly on the mode of presentation. Cell-bound protein given i.v. leads to the production of T_{s}. We have previously shown that the spleen is required for at least some forms of T_{s} activation (35). As many i.v. protein-modified LC will travel to the spleen, we think it likely that this blood-borne presentation will stimulate the production of T_{s} or suppressive factors which can leave the spleen to suppress T_{DH} in lymph nodes. We do not know whether splenic macrophages are important in this process, but it has been suggested by Feldmann and Kontiainen (36) and by Benacerraf and Germain (37) that bypass of macrophage processing may be an important step in triggering of T_{s}.

Thus, we have described a relatively simple method for inducing both T-cell-
mediated immunity and tolerance (plus suppression) to protein antigens in a mouse model. \( \text{T}_\alpha \) can be induced with relatively low doses of membrane-bound antigen, as opposed to other systems where high doses of deaggregated globulins (38) are required for \( \text{T}_\alpha \) induction. The use of membrane-bound antigens as tolerogens may also circumvent problems recently described by Parks et al. (39) in the human \( \gamma \)-globulin system where different preparations of deaggregated material induced very variable \( \text{T}_\alpha \) activities (in some cases no demonstrable \( \text{T}_\alpha \)). The use of membrane-associated protein antigens may also allow the induction of tolerance in systems where soluble proteins are not effective tolerogens. In this regard, we have obtained recent evidence (S. D. Miller and J. M. Chiller, unpublished observations) that human \( \gamma \)-globulin coupled LC are effective tolerogens for both CMI and antibody production in BALB/c mice, which are historically difficult to tolerize even with high doses of deaggregated \( \gamma \)-globulins (40, 41). It is hoped that eventually the use of antigen-modified autologous lymphoid cells as tolerogens can be used clinically to modulate harmful T-cell-mediated immune responses such as autoimmune states.

Summary

A mouse model of cell-mediated immunity (CMI) and tolerance to protein antigens horse gamma globulin (HoGG) and cytochrome (Cyt C) was investigated. A reliable CMI response as measured in vivo by ear swelling or by an in vitro T-cell proliferation assay could be induced by one of two methods: (a) sensitization by antigen-complete Freund's adjuvant in the base of the tail, or (b) sensitization by s.c. injection of antigen coupled to syngeneic lymphoid cells. The in vivo response exhibited characteristic CMI parameters, delayed kinetics, and transfer by viable T cells.

Prior i.v. injection of HoGG-modified lymphoid cells (HoGG-LC) or Cyt C-LC before sensitization resulted in a rapidly induced, dose-dependent, antigen-specific suppression of both in vivo and in vitro manifestations of the CMI response. In addition, tolerance in this system was transferrable by an antigen-specific suppressor T cell (\( \text{T}_\alpha \)). The \( \text{T}_\alpha \) were found to diminish the in vivo ear swelling reaction in recipient animals, but had no effect on the in vitro T-cell proliferative response of the recipients. In contrast to the rapid development of tolerance in donor mice (phenotypic tolerance), transferrable \( \text{T}_\alpha \) were first demonstrable 4–7 d posttolerization. This latter result indicates that at least two mechanisms of tolerance are operative in this system: the rapid induction of clone inhibition of reactive T cells and the slower induction of \( \text{T}_\alpha \).

These results indicate again that the mode of antigen presentation is crucial in determining the immunologic outcome. In these experiments, cell-bound proteins injected subcutaneously led to delayed hypersensitivity while the same antigens injected intravenously led to tolerance.

These results are considered in the light of recent experiments which show that T cells recognize antigens on cells in association with major histocompatibility complex products. We believe the following pathways are involved. In sensitization via subcutaneous injection of HoGG-LC, antigen reaches the lymph node via lymphatic pathways which lead to immunogenic macrophage-associated presentation and the activation of delayed hypersensitivity T cells (TDH). In tolerization via intravenous injection of HoGG-LC, antigen (a) reaches the lymph node via the blood, probably directly meeting the TDH, preventing its subsequent activation by immunogenic
HoGG (clone inhibition) and (b) reaches the spleen, also via the blood, activating suppressor T cells.

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