MECHANISM OF T-CELL HELP IN THE IMMUNE RESPONSE TO SOLUBLE PROTEIN ANTIGENS

I. Evidence for In Situ Generation and Action of T-Cell-Replacing Factor during the Anamnestic Response to Dinitrophenyl Keyhole Limpet Hemocyanin In Vitro*

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In the humoral immune response to most antigens studied so far, the presence of helper T cells is required for the generation of antibody-forming cells from precursor B cells. In terms of the classical carrier-hapten systems described by Mitchison (1), B cells specific for a hapten can only become antibody-forming cells if helper T cells react with antigenic determinants on the carrier molecule that the hapten is linked to. This finding has later on led to the hypothesis that the close proximity brought about by an antigen "bridge" between B cell and T cell is the prerequisite for T-B cooperation (2). Under this assumption, several concepts have been developed to explain the nature of T-cell help itself. Among these are: the focussing of antigen by the T cell onto the B cell (2, 3), a membrane interaction between the two cells (4), and the production of mediators, antigen specific (5-7) or nonspecific (4, 7-14), by the antigen-stimulated T cell. In vitro studies have shown that T-cell-replacing factor (TRF), a nonantigen-specific mediator produced by activated T cells (15), can replace T cells in primary and secondary immune responses to heterologous erythrocytes (15, 16). The action of TRF and similar factors described by other authors (4, 7-14) has so far been demonstrated by restoring the immune response to heterologous erythrocytes in T-cell-deprived cultures by addition of preparations containing the factor. On the other hand, the experiments reported by Hartmann (17) have shown that T cells activated to erythrocyte antigens in vivo do, upon antigenic challenge in vitro, not only restore the response of B-cell cultures to the epitopes of the same erythrocytes, but also to those of erythrocytes from another species added simultaneously to the same cultures. Although this co-stimulation is best explained by the in situ generation and action of TRF, the function, and indeed the presence of this mediator in complete spleen cell cultures during the immune response remains to be demonstrated.

In order to study this question under the most stringent conditions, we chose the anamnestic IgG reaction to soluble carrier-hapten conjugates in vitro. The pronounced T-cell dependence of the IgG response to such a conjugate and the possibility to stimulate T and B cells separately with carrier and hapten determinants make it possible to investigate the action of TRF in the immune response to soluble protein antigens.

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1 Abbreviations used in this paper: BSS, Hanks' balanced salt solution; DNP, dinitrophenyl; FDNB, fluorodinitrobenzene; GPC, guinea pig complement; HRBC, horse red blood cells; KLH, keyhole limpet hemocyanin; OVA, ovalbumin; PFC, plaque-forming cells; TRF, T-cell-replacing factor.
Materials and Methods

**Mice.** DBA/2 mice were obtained from Bomholtgard, Ry, Denmark. Mice of both sexes between 6 wk and 6 mo of age were used.

**Antigens.** Soluble protein antigens were Keyhole limpet hemocyanin (KLH), dinitrophenyl KLH (312 DNP groups per 10^6 mol wt), DNPBGG (310 DNP groups per 10^6 mol wt), all from Calbiochem, San Diego, Calif., and DNPOVA (194 DNP groups per 10^6 mol wt). The latter was prepared as described in reference 18 by reacting Ovalbumin (OVA) (Sigma Chemical Co., St. Louis, Mo.) in 0.05 M NaHCO₃ with fluorodinitrobenzene (FDNB) in dioxane. After exhaustive dialysis against water, the product was lyophilized. The hapten Gly₂-DNP was prepared by reacting Gly-Gly in 0.05 M NaHCO₃ with an excess of FDNB in ethanol according to Sanger (19).

**Immunizations.** Mice were primed 6-14 wk before use with an intraperitoneal (i.p.) injection of 200 μg alum-precipitated protein and 10^9 Bordetella pertussis organisms (Behringwerke, Marburg-Lahn, W. Germany). Boosting was performed by an i.p. injection of 20 μg soluble protein. DNPKLH-primed mice were boosted 7 days before use. KLH-specific helper cells were taken 3 days after boosting of KLH-primed mice; in agreement with other authors (20), better helper activity could be obtained than after a 7-day boost.

**Tissue Culture.** Spleen cell cultures were prepared as described by Mishell and Dutton (21) and cultured in Eagle's minimal essential medium (MEM) for normal cultures (Microbiological Associates, Inc., Bethesda, Md.). 5 × 10^5 nucleated cells in 0.5 ml of culture medium containing 5 × 10^−2 M 2-mercaptoethanol, 5% fetal calf serum (Armour Pharmaceutical Co., Chicago, Ill.), and supplemented as given in reference 21 were seeded into tissue culture plates (Costar, Cambridge, Mass.) with 24 flat-bottom holes (15-mm diameter). One drop of a nutritional cocktail (21) was added on each day of culture. Cultures were harvested at the times indicated, washed once in Hanks' balanced salt solution (BSS) and resuspended in the appropriate dilutions for the plaque assay.

**Anti-Thy 1.2 Treatment of Spleen Cells.** Anti-Thy 1.2 ascitic fluid was prepared according to Raff (22) by 6 wk i.p. injections of 10^9 CBA thymus cells into AKR mice (The Jackson Laboratories, Bar Harbor, Maine). Together with the last injection, 0.1 ml of a 1:10 dilution of freshly harvested mouse Krebs II ascites cells were injected. The ascitic fluid was collected 1 wk later. For removal of T cells, 6 × 10^5 spleen cells were kept in 1 ml of this fluid at a dilution of 1:3 in medium for 30 min on ice. Cells were washed and resuspended in the same volume of agarose-absorbed rabbit complement at a dilution of 1:5 in medium, incubated for 45 min at 37°C, washed twice, resuspended in medium, and adjusted to the appropriate concentration.

**Assay for Anti-DNP PFC.** The Jerne hemolytic plaque assay was used as modified by Mishell and Dutton (21). Horse red blood cells (HRBC) were sensitized with 2,4,6-trinitrobenzene sulfonic acid as described by Rittenberg and Pratt (23). HRBC were always taken from the same horse and showed only negligible background plaque-forming cells (PFC). After plating, the slides were covered with BSS and incubated for 90 min at 37°C. The BSS was then replaced by guinea pig complement (GPC) diluted 1:15 in BSS and incubated for another 90 min. At this point, IgM-PFC were recorded and the slides reincubated with a 1:75 dilution of rabbit anti-mouse Ig serum (Behringwerke) in diluted GPC for 45 min. Again, the hemolytic plaques were counted. The difference between the two counts, i.e., the "indirect" PFC, will be referred to as IgG-PFC in this paper. This evaluation seems reliable to us because (a) the number of IgG-PFC in positive cultures was always about 10 times higher than the number of IgM-PFC, and (b) cultures from KLH-primed cells, upon incubation with DNPKLH, showed few IgM but absolutely no IgG-PFC. The number of recovered nucleated cells was determined in a Coulter Counter (model F₄, Coulter Electronics, Inc., Hialeah, Fla.), using Zaponin (Coulter Electronics, Inc.) to lyse red cells. Results will be given as anti-DNP IgG-PFC per million recovered nucleated cells (IgG-PFC/10⁶).

**Results**

Recently, North and Askonas have described an in vitro system (24) in which very high responses to DNPKLH can be obtained. Just as in the anamnestic response in vivo, the vast majority of anti-DNP-PFC induced in these cultures produce IgG antibodies, whereas IgM-PFC represent only 10-15% of the response. As the IgG response is known to be even more sensitive to the removal of T cells than is the primary, predominantly IgM response (25), we have used this
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system for a study on the production and action of TRF in the response to soluble protein antigens. The same immunization schedule was used as described by North and Askonas (24), and tissue cultures were set up as described in Materials and Methods. Fig. 1 shows a typical dose-response profile of spleen cells from DNPKLH primed and boosted mice after 5 days of culture. The optimal concentration of DNPKLH varied between 0.01 and 0.1 \( \mu \)g DNPKLH per culture, and the response was sometimes as high as 30,000 IgG-PFC/10^6. Thus, the properties of the system as reported by North and Askonas could be fully reproduced.

To characterize this response as a classical carrier-hapten system, it was investigated if the recognition of the DNP moiety by the B cells could be blocked by free hapten and if, on the other side, the recognition of the KLH moiety by helper T cells could be blocked by free carrier. Fig. 2 shows that, quite in agreement with other authors who employed heterologous carrier-hapten conjugates (26-30), we found that the response could be easily blocked by the addition of free hapten. The presence of 4 \( \mu \)g Gly2DNP resulted in a 85% reduction of the response to 0.01 \( \mu \)g DNPKLH, and with 16 \( \mu \)g Gly2DNP, background levels were reached. In parallel experiments it was found that the concentrations of Gly2DNP used were not toxic for the cultures, as the primary response to sheep red blood cells (SRBC) was unimpaired, and higher doses of Gly2DNP were required to block higher concentrations of DNPKLH (data not shown).

On the other hand, we were unable to block T-cell help by increasing amounts of "free" carrier (Fig. 3). At 4 \( \mu \)g KLH per culture, a concentration 400-fold higher than that of DNPKLH, we observed even a stimulation of the response, and at higher concentrations the values were still at control levels without KLH. Here it seems obvious that the KLH-primed helper cells do not have to recognize KLH on the same molecule that binds to and stimulates the B cells via the DNP-specific antigen receptor to exert their helper function. This point was
FIG. 2. Inhibition of the IgG response to DNPKLH by free hapten. Spleen cells from mice primed and boosted with DNPKLH were cultured in the presence of 0.01 μg DNPKLH and increasing amounts of the hapten Gly₂DNP. Anti-DNP IgG-PFC were tested on day 5 of culture.

FIG. 3. Failure to inhibit the IgG response to DNPKLH by free carrier. Spleen cells from mice primed and boosted with DNPKLH were cultured in the presence of 0.01 μg DNPKLH and increasing amounts of KLH. Anti-DNP IgG-PFC were tested on day 5 of culture.
now tested by presenting the hapten to the B cell on a heterologous carrier while stimulating the KLH-reactive helper cells by free KLH. Fig. 4 shows such an experiment. Spleen cell cultures from mice primed and boosted with DNPKLH were cultured with increasing amounts of KLH in the presence (●—●) or absence (○—○) of 0.1 μg DNPBGG. The response to 0.1 μg DNPKLH in the same experiment is included for comparison. Cultures were assayed for anti-DNP IgG-PFC on day 5.

Fig. 4. Stimulation of the anamnestic anti-hapten IgG response by simultaneous addition of a heterologous carrier-hapten conjugate and free homologous carrier. Spleen cells from mice primed and boosted with DNPKLH were cultured with increasing amounts of KLH in the presence (●—●) or absence (○—○) of 0.1 μg DNPBGG. The response to 0.1 μg DNPKLH in the same experiment is included for comparison. Cultures were assayed for anti-DNP IgG-PFC on day 5.
### Table I

**Stimulation of the Anamnestic Anti-Hapten IgG Response by Simultaneous Addition of a Heterologous Carrier-Hapten Conjugate and Homologous Carrier**

|                      | Exp. I | Exp. II | Exp. III |
|----------------------|--------|---------|----------|
| No addition          | 189    | 106     | 292      |
| 0.1 μg DNPKLH        | 11,533 | 6,684   | 27,798   |
| 0.1 μg DNPBGG        | 528    | 250     | 674      |
| 1 μg KLH             | 747    | 433     | 901      |
| 0.1 μg DNPBGG + 1 μg KLH | 6,855 | 5,764   | 11,400   |
| 1 μg DNPOVA          | ND     | ND      | 869      |
| 0.1 μg DNPOVA + 1 μg KLH | ND    | ND      | 6,889    |

Spleen cells from DNPKLH-primed and boosted mice were cultivated in the presence of a heterologous DNP-protein conjugate with or without simultaneous addition of the homologous carrier KLH. Results are given as anti-DNP IgG-PFC/10⁶ recovered cells tested on day 5 of culture.

ND, not done.

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Fig. 5. Dependence of in situ TRF production on carrier-primed helper T cells. Spleen cells from DNPKLH-primed and boosted mice were treated with rabbit complement (upper frame) or with anti-Thy 1.2 serum and complement (lower frame). In the second group, either 5 × 10⁶ cells were cultured alone or 10⁶ cells were co-cultivated with 4 × 10⁶ spleen cells from KLH-primed and boosted mice or with 4 × 10⁶ spleen cells from normal mice. Antigen concentrations used were: DNPKLH, DNPBGG, and DNPOVA: 0.1 μg/culture; KLH: 1 μg/culture. Results are expressed as anti-DNP IgG-PFC/10⁶ recovered cells tested on day 4 of culture, corrected for the proportion of DNP-primed B cells.
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**Table II**

*Dependence of In Situ TRF Production on Carrier-Primed T Cells*

| Number and treatment of DNPKLH-primed and boosted cells* | Number of KLH helper cells† | Antigen(s) in culture§ | IgG-PFC/10⁶ day 4 |
|-----------------------------------------------------------|-----------------------------|-----------------------|------------------|
| 5 × 10⁶ C'                                                | –                           | DNPKLH                | 145              |
| 5 × 10⁶ C'                                                | –                           | DNPOVA                | 153              |
| 5 × 10⁶ anti-Thy + C'                                     | –                           | DNPKLH                | 88               |
| 5 × 10⁶ anti-Thy + C'                                     | –                           | DNPKLH                | 87               |
| 5 × 10⁶ anti-Thy + C'                                     | –                           | DNPBGG                | 85               |
| 5 × 10⁶ anti-Thy + C'                                     | –                           | DNPOVA                | 184              |
| 5 × 10⁶ anti-Thy + C'                                     | –                           | KLH                   | 73               |
| 5 × 10⁶ anti-Thy + C'                                     | –                           | DNPBGG + KLH          | 86               |
| 5 × 10⁶ anti-Thy + C'                                     | –                           | DNPOVA + KLH          | 153              |
| 4 × 10⁶ anti-Thy + C'                                     | DNPKLH                      |                       | 2,690||
| 4 × 10⁶ anti-Thy + C'                                     | DNPBGG + KLH                |                       | 3,417||
| 4 × 10⁶ anti-Thy + C'                                     | DNPOVA + KLH                |                       | 1,739||
| –                                                         | 5 × 10⁶ DNPKLH               |                       | 0                |
| –                                                         | 5 × 10⁶ DNPBGG + KLH         |                       | 0                |
| –                                                         | 5 × 10⁶ DNPOVA + KLH         |                       | 0                |

* Spleen cells from DNPKLH-primed and boosted mice were treated with rabbit complement (C') or with anti-Thy 1.2 serum plus complement.
† Spleen cells from mice primed and boosted with KLH.
§ DNPKLH, DNPBGG, DNPOVA: 0.1 μg/culture; KLH: 1 μg/culture.
|| Corrected for the proportion of DNP-primed B cells.

boosted spleen cells with anti-Thy 1.2 serum and complement before culture abolished not only the response to DNPKLH but also the helper effect induced by free KLH for B cells stimulated with DNPBGG or DNPOVA. Furthermore, the addition of KLH-specific helper cells reconstituted both the response to DNPKLH and to the combinations DNPBGG + KLH and DNPOVA + KLH, whereas the addition of unprimed spleen cells had no effect (Fig. 5). In both experiments, helper cells alone gave no response (data in Fig. 5 not shown). These experiments demonstrate that antigen-primed T cells can, upon antigenic stimulation, produce a nonspecific mediator, presumably identical with TRF, which helps B cells stimulated by a soluble protein antigen to become 7S antibody-forming cells.

**Discussion**

It is well documented by numerous reports that in vitro immune responses to the T-cell-dependent antigens of heterologous erythrocytes can be obtained in the absence of T cells if TRF (8) or similar nonantigen-specific mediators produced by activated T cells (4, 9–14) are added to the cultures. It has been demonstrated that TRF is produced by T cells (15), that carry the Ly phenotype characteristic for helper T cells (31), and that at least functionally very similar mediators are produced by antigen-educated T cells upon rechallenge (7, 9, 10, 13). There are, however, no reports demonstrating the actual involvement of
TRF in the in vitro immune response of entire mouse spleen cell populations. Carrier-hapten systems as the ones employed in this report (24) are well suited to study this question, because it is possible to stimulate the carrier-specific cells by unhaptened carrier without stimulating the hapten-reactive B cells. Hapten-free homologous carrier or heterologous carrier-hapten conjugates have been used by several authors (26-30) in attempts to analyze selectively the reactivity of helper T cells and hapten-specific B cells. There is general consensus that the anti-hapten response can be inhibited by the hapten on a heterologous carrier (26-30). Correspondingly, our experiments show that during the anamnestic response to DNP KLH in vitro the hapten reactive B cells can be blocked by Gly2DNP (Fig. 2). Regarding the helper T cells, Bluestein and Pierce were able to inhibit the primary immune response to TNP-φX by adding unhaptened φX (26). This seems to be in contrast to the results presented here. In an anamnestic anti-DNP KLH immune response, KLH-specific helper cells still provide their helper function in the presence of a 1,000 times higher concentration of KLH than that of the conjugate DNP KLH (Fig. 3). This seeming discrepancy can be reconciled within the frame work of the original Mitchison-Rajewsky model (2) as follows: in case of the primary immune response to TNP-φX (26), relatively few φX-specific helper cells are present in the cultures. Only if T and B cells are actually linked by the antigen TNP-φX will the B cells receive enough of the (nonantigen-specific) mediator (TRF) (8), which is produced by the helper cell that has reacted with the carrier determinants. The anamnestic response to DNP KLH in vitro, however, is about 100-fold higher in terms of anti-DNP PFC than is the primary response to TNP-φX. Quite obviously, and as is shown by the experiments described in this paper, there are so many KLH-specific helper cells present in these cultures that enough helper factor is produced to reach even those B cells that are not linked to them by an antigen bridge. This notion was verified by applying experimental conditions which exclude an antigen bridge between DNP-reactive B cells and KLH-specific T cells; DNP BGG or DNOVA are able to stimulate the antibody response of the DNP-specific B cells if unhaptened KLH is added simultaneously to stimulate the numerous primed helper T cells (Fig. 4 and Table I). The positive outcome of this experiment is in agreement with the observations of Hartmann (17) in which the in situ action of a nonantigen-specific helper factor produced by "educated" T cells was demonstrated in the primary immune response to heterologous red blood cells. A similar experiment was described by Marrack and Kappler (32) in which KLH-primed spleen cells, upon stimulation with this antigen, helped SRBC-stimulated B cells in the same culture to become IgM hemolysin-producing cells. Kishimoto and Ishizaka (33) described an in vitro system consisting of rabbit mesenteric lymph node cells primed with DNP-ragweed. It was found that stimulation of rag-sensitized cells with free rag enhanced anti-DNP IgG and IgE responses to DNP-unrelated carrier conjugates. Cell-free supernates obtained from cultures of primed lymph node cells incubated with free carrier also showed this enhancing effect. These systems are comparable to the one employed in this paper with respect to the presence of primed helper cells, and just as in our system, the results are most easily explained by assuming a "surplus" production of a nonantigen-specific T-cell
helper factor, presumably TRF, which under the confined conditions of the in vitro system bypasses the need for T-B bridging.

These data are also in agreement with reports on the production of TRF-like supernates upon stimulation of T cells educated to KLH (7, 10, 13) or heterologous erythrocytes (7, 9) with the respective antigens. Our data demonstrate, furthermore, the ability of such a mediator induced by antigen to restore the immune response to a soluble protein antigen and show the presence and action of this factor in untreated spleen cell cultures from immunized mice without the addition of educated T cells. It has been postulated that for the reconstitution of the primary (7) and the secondary (32) immune responses to soluble protein antigens in the absence of T cells, an antigen-specific helper factor is required in addition to the nonspecific mediator. In our system, using cells from primed and recently boosted mice, this was obviously not the case; the subsequent publication (34) will further strengthen this point.

The concept that the in situ generation of TRF during the anamnestic immune response to DNPKLH is a function of the KLH-specific helper cells was investigated in T-cell depletion and reconstitution experiments (Fig. 5, Table II). Indeed, the observed helper effect induced by free carrier for the hapten on a heterologous carrier was completely abolished by treatment with anti-Thy 1.2 + complement. It could be fully reconstituted by carrier-primed but not by normal spleen cells. All these data suggest that during the anamnestic immune responses to haptenated soluble protein antigens in vitro, carrier-specific helper cells produce a nonantigen-specific mediator such as TRF, which can act on hapten-specific B cells that are stimulated by a heterologous carrier-hapten conjugate. Analogous in vivo experiments have led to conflicting reports. Several authors reported negative results (35, 36).

On the other hand, Hamaoka et al. (37) found a stimulation of anti-hapten IgE responses in vivo after transfer of DNP-Ascaris-primed mouse cells and challenge with DNP-KLH in the presence of free Ascaris carrier. A very weak anti-hapten IgG response was also observed provided additional Ascaris-primed helper cells were transferred.

The difficulties encountered in the in vivo experiments may be due to diffusion and/or inactivation of TRF. The conditions for reaching and maintaining sufficient local levels of TRF will be certainly less favorable in vivo than in vitro. Nevertheless, the experiments reported in this communication strengthen the view that TRF is a physiological signal by which T cells help in the generation of antibody-forming cells to T-dependent antigens.

Alternative explanations cannot as yet be formally excluded. For instance, there could be an antigen nonspecific and direct membrane interaction between T and B cells. This could be brought about by fortuitous interaction of independently stimulated T and B lymphocytes or by the close proximity of both cell types assembled on a macrophage. However, our data presented in the subsequent publication (34) make this very unlikely.

Summary

The involvement of a nonantigen-specific T-helper factor in the anamnestic immune response to dinitrophenyl keyhole limpet hemocyanin is demonstrated.
employing cultures of unseparated spleen cell populations. In such cultures of primed and boosted spleen cells, a good IgG anti-DNP response could be obtained if the hapten was presented on a heterologous carrier, provided that the homologous carrier was added simultaneously. T-cell depletion and reconstitution experiments show that such a factor, presumably identical with T-cell-replacing factor, is produced by primed helper cells upon rechallenge and helps primed B cells, stimulated by soluble heterologous carrier hapten conjugates, to become IgG-secreting cells.

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