GENERATION OF T-HELPER CELLS IN VITRO

II. Analysis of Supernates Derived from T-Helper Cell Cultures*

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Antibody production in response to thymus-dependent antigens results from a complex series of cellular interactions in which antibody-forming cell precursors (bone marrow derived or B lymphocytes) proliferate and differentiate to synthesize specific antibody. The regulation of this response involves cellular and humoral interactions in which thymus-derived lymphocytes (T cells) act as "helpers" as well as "suppressors" in several different systems (1–8). The nature of the regulatory stimuli provided by T cells has not been established. There is evidence that T cells produce mediators that regulate B-cell responses (reviewed in reference 9). It has been suggested that immunoglobulin-like molecules or products related to the major histocompatibility locus are the likely candidates for T-cell regulatory factors (9–10). Immunoglobulin and products of the major histocompatability locus have been shown to have both facilitatory (11–22) and suppressive (23–27) as well as antigen-specific (11–17, 24–27) and nonspecific (15–24) activities on B-cell responses in various systems.

In the preceding paper (28) we describe an in vitro culture system based on that originally proposed by Kontianin and Feldmann and Erb and Feldmann (29–32) using purified thymocytes and peritoneal exudate macrophages cultured under conditions selected to generate T-helper cells. The present study is an examination of supernatant factors derived from in vitro generated T-helper cell cultures. The supernates from such cultures are active in enhancing B-cell anti-hapten responses when assayed in vitro on hapten-primed spleen cell cultures. The data indicate that the active moiety is a nondialyzable, trypsin-sensitive product(s) which is removed by preabsorption with alloantisera but not anti-immunoglobulin sera. Supernates display both antigen-specific and nonspecific properties which can be distinguished by supernatant dosage, antigen requirements, and the need for splenic adherent accessory cell involvement.

Materials and Methods

The mice strains, antigens, cell preparations, and culture conditions for the in vitro induction and assay of T-helper cells were described in the previous paper (28). Anti-Thy-1.2 sera and complement treatment and macrophage depletion of spleen cell preparations were performed as previously described (28).

Harvest of Supernates. Thymocyte-macrophage cultures primed with carrier protein under conditions optimal for the generation of T-helper cells (28) were harvested after 4 days. The cells were separated by centrifugation at 300 g for 7 min and assayed for helper cell activity The supernate was centrifuged at 900 g for 10 min and 10,000 g for 20 min, passed through a 0.22 μm filter (Millipore Corp , Bedford, Mass.), and stored at $-20°$C until used.

Assay of Supernatant Activity. Supernates were assayed for their capacity to enhance the anti-dinitrophenyl (DNP)[1] plaque-forming response (to DNP-protein conjugates) of cultured spleen cells from mice primed with 25 μg DNP-Ficoll 3-4 mo previously. Supernatant culture fluid in doses indicated, culture medium, 1 5 $\times$ 10^7 spleen cells, and antigen (1 μg DNP-keyhole limpet hemocyanin [KLH] or 5 μg DNP-fowl gamma globulin [FγG]) were added in that order and cultured in 1.0 ml vol in Marbrook-Diener chambers. After 4 days, the cultures were harvested, and the specific anti-DNP-PFC response was assayed with the hapten inhibition hemolytic plaque technique described in the previous paper (28).

Immunoglobulins. BALB/c mice carrying the plasmacytomas TEPC 183 (IgMκ), MOPC 104E (IgMλ), and HOPC1 (IgG-$\gamma_{2a\lambda}$) were obtained from Litton Bionetics, Kensington, Md., through the courtesy of Dr Michael Potter (National Institutes of Health). IgM preparations were purified by Sephadex G-200 chromatography of the sera and subsequent preparative electrophoresis of the void volume peak. Preparative electrophoresis was performed with a column electrophoretic apparatus (Uniphor 9300, LKB Instruments, Inc., Rockville, Md.) containing a Sephadex G-25 column bed equilibrated with 0 1 M Tris buffer, pH 8.6. The IgG myeloma protein was purified by Sephadex G-200 chromatography of a 35% saturated ammonium sulfate precipitate of the sera and subsequent DEAE column chromatography The 7s globulin fraction of normal mouse sera was obtained by Sephadex G-200 chromatography. For absorption of antisera, these preparations were conjugated to cyanogen bromide-activated Sepharose 4B (33)

Antisera Antisera to purified mouse myeloma proteins were produced in goats. 10 mg of protein in complete Freund's adjuvant were given subcutaneously followed by another 10 mg in saline 4 wk later The goats were bled 10 days after the second inoculation. Injections were repeated as necessary.

Antisera to IgM (TEPC 183 IgMκ and MOPC 104E IgMλ) were rendered μ-chain specific by absorption with Sepharose 4B-conjugated preparations of alpha-2 macroglobulin (obtained during the preparative electrophoresis used in the purification of the IgM antigens), IgGλ (HOPC1), 7s globulins, and finally at 4°C with mouse thymocytes (two thymuses per ml). Goat anti-TEPC 183 IgMκ serum was rendered specific for $\mu\kappa$-determinants by the above procedure except that the 7s globulin absorption was omitted

Antisera to IgGλ (HOPC1) were rendered γ-chain specific by absorption with Sepharose 4B-conjugated preparations of TEPC IgMκ, MOPC IgMλ, and void volume material from Sephadex G-200 chromatography of normal mouse sera, as well as by absorption with mouse thymocytes.

A polyvalent anti-mouse immunoglobulin serum was prepared by pooling antisera to IgM and IgG and absorbing with alpha-2 macroglobulin and mouse thymocytes The specificity and absorptions of the anti-immunoglobulin sera were checked by immunoelectrophoresis and double diffusion in agar.

Preparations of purified antibody to immunoglobulins were obtained by absorption of the specific antisera to columns containing the appropriate immunoglobulin conjugated to Sepharose 4B Absorption was done at 4°C and followed by washing the columns with 0.2 M borate 0.15 M saline buffer pH 8.0. The absorbed antibodies were eluted with 3 M NaSCN (34). The material eluted in 3 M NaSCN was dialyzed against borate saline buffer, concentrated, and conjugated to cyanogen bromide-activated Sepharose 4B (33).

Alloantisera were produced against BALB/c (H-2^d) and CBA/J (H-2^k) mice by reciprocal immunization. CBA anti-BALB/c (anti-H-2^d) and BALB/c anti-CBA (anti-H-2^k) were produced by immunizing the mice intraperitoneally with six biweekly injections of 10^7 pooled thymus and spleen cells from 3- to 5-wk-old mice Mice were bled 10-12 days after the last injection Globulin

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[1] *Abbreviations used in this paper* AEF, allogeneic effect factor, ATC S/N, supernatant culture media derived from in vitro activated thymus-derived cells; DNP, dinitrophenyl; FγG, fowl gamma globulin; KLH, keyhole limpet hemocyanin; PBS, phosphate buffered saline; PFC, plaque-forming cell

fractions of the two alloantisera were precipitated in 45% saturated ammonium sulfate, dialyzed against borate-saline buffer, and conjugated to cyanogen bromide-activated Sepharose 4B.

Absorption of Supernatant Fluid with Sepharose 4B Conjugates The following Sepharose 4B-conjugated proteins, and the volume of Sepharose 4B (7–9 mg of protein conjugated to each ml of Sepharose 4B) were used to absorb 8 ml of supernate: KLH, 4 ml; FγG, 4 ml; polyvalent anti-mouse immunoglobulin antibodies, 12 ml; anti-mouse $\mu\kappa$-antibodies, 14 ml; anti-mouse μ-chain antibodies, 10 ml; anti-mouse γ-chain antibodies, 10 ml; CBA anti-BALB/c globulin, 22 ml; BALB c anti-CBA globulin, 21 ml. Preimmune normal goat globulin, CBA, and BALB/c mouse globulin fractions conjugated to Sepharose 4B were prepared as controls.

Sepharose preparations in 0.01 M PO_4, 0.15 M NaCl, pH 7.2, (phosphate-buffered saline, PBS), were packed into vertical 10- or 25-ml pipettes fitted with tubing and a glass wool plug. Supernatant fluid was absorbed to the various Sepharose 4B columns by slow passage (at least 2 h) at 4°C through the column and eluted with PBS. Supernatant fluid was filtered and assayed immediately. Efficacy of immunoglobulin absorption was monitored by adding trace amounts of the appropriate ^{125}I-labeled immunoglobulin to the supernate. After absorption, columns were washed with 3 M NaSCN and then washed and stored in PBS containing 0.1% Na azide. Before use they were washed extensively with sterile PBS. All absorption experiments were performed three times (except the anti-γ-chain absorption which was performed once).

Trypsin and Periodate Treatment of Supernatant Fluid. Trypsin (2 × crystallized, Type III from bovine pancreas) (Sigma Chemical Co., St. Louis, Mo.) insolubilized on Sepharose 4B (33) was reacted with two parts by volume of supernate in closed containers, pH 7.4–7.8, at 37°C for 16 h on a rocking platform Control supernatant fluid was incubated in parallel with unconjugated Sepharose 4B. Under these conditions ^{125}I-labeled protein (IgMκ) added to the digestion mixture was reduced to a 30–52% precipitable material in 10% trichloroacetic acid, whereas the control incubated preparation was unaffected (90–95% precipitable). Trypsin-Sepharose conjugates were used once and discarded.

Periodate treatment of the supernatant fluid was performed by dialysis against 0.01 M sodium periodate in PBS, pH 7.2, (8 ml supernate per liter of dialysis fluid) in the dark at 4°C for 18 h (35). Periodate-treated supernate was then dialyzed against several changes of PBS, filtered, and the treated supernate assayed. Control supernate was dialyzed against PBS in parallel.

Results

Capacity of Supernatant Culture Fluid Derived from Helper Cell Cultures to Enhance the Anti-hapten Responses of Spleen Cell Cultures to Hapten Carrier Conjugates. Supernatant fluid, harvested after 4 days from thymocyte-macrophage cultures primed with carrier under conditions optimal for generating helper cells, is active in enhancing the anti-DNP-plaque-forming cell (PFC) response of cultured spleen cells from hapten-primed mice. This is shown in Fig. 1. Several points emerge from this analysis. The dose of supernate used influences the anti-hapten response obtained. The response to a given hapten-carrier conjugate is greatest and optimal at lower doses of supernatant fluid from helper cell cultures which had been primed with the homologous carrier. At higher supernatant doses, significant enhancement of DNP-PFC responses is seen in the absence of hapten-carrier conjugates. This nonspecific enhancement is even more pronounced in the presence of hapten coupled to a noncross-reacting carrier, that is, a conjugate not used in generating the supernate.

The enhancement of PFC responses seen at higher supernatant doses was not confined to DNP-specific PFC responses. The number of PFC's formed against TNP-SRBC in the presence of 5 × 10^{-4} M DNP-L-lysine, and the number of PFC's formed against unconjugated SRBC were also increased in cultures with the greater doses of supernate. Supernatant doses above 0.5 ml in a 1.0 ml culture resulted in lower and variable cell recovery from the cultures, and,
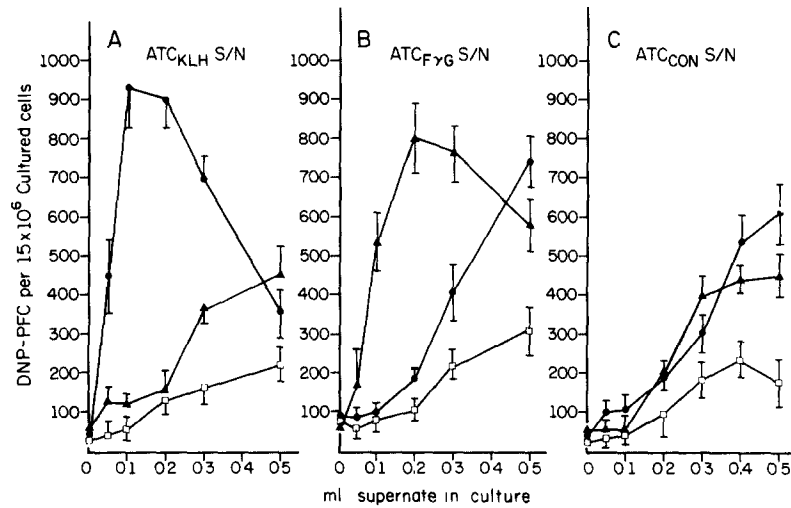
Fig. 1. Enhancement of splenic DNP-PFC responses by supernates derived from thymocyte-macrophage cultures. Supernatant fluids were added to spleen cell cultures in the doses indicated together with hapten-protein conjugates; DNP-KLH, (●—●); DNP-FγG, (△—△); or without additional antigen, (□—□). Results indicate the DNP-PFC response ± SD of triplicate cultures receiving supernate derived from thymocyte-macrophage cultures primed with either (A) KLH (ATC$_{KLH}$S/N), (B) FγG (ATC$_{F\gamma G}$ S/N), or (C) no priming carrier (ATC$_{CON}$ S/N).

therefore, only experiments where cell culture recoveries were consistent are reported.

The Effect of Helper Cell Culture Supernates on Macrophage-Depleted and T-Depleted Spleen Cell Cultures. The activity of supernates derived from thymocyte-macrophage cultures is ultimately expressed as an enhanced anti-DNP-PFC response by cultured, hapten-primed spleen cells. To determine whether the active factor(s) acted directly on B cells or require additional splenic T-cell or macrophage interaction, we tested supernates on macrophage-depleted or T-depleted spleen cell cultures. The results are shown in Figs. 2 and 3. Efficacy of T-cell depletion by anti-Thy-1.2 serum and complement treatment or adherent cell depletion by Sephadex G-10 column incubation was assessed by membrane marker studies and by the primary in vitro response of the treated cultures to SRBC, a response known to require T cells (36) and macrophages (37).

The response of spleen cell cultures to the supernate is not effected by depletion of T cells (Fig. 2). In fact, in several experiments, the response was greater than expected from the increased number of potentially responsive B cells in these cultures (as determined either by the response to DNP-Ficoll or by the calculated enrichment due to depletion of T cells by anti-Thy-1.2 sera and complement treatment).

Macrophage depletion of recipient spleen cell cultures caused them to have a different response to supernates than normal spleen cell cultures (Fig. 3). The nonspecific enhancement of anti-DNP-PFC responses seen at higher supernatant doses is maintained in macrophage-depleted spleen cell cultures. The enhancement in the carrier-specific response usually seen in normal spleen cell
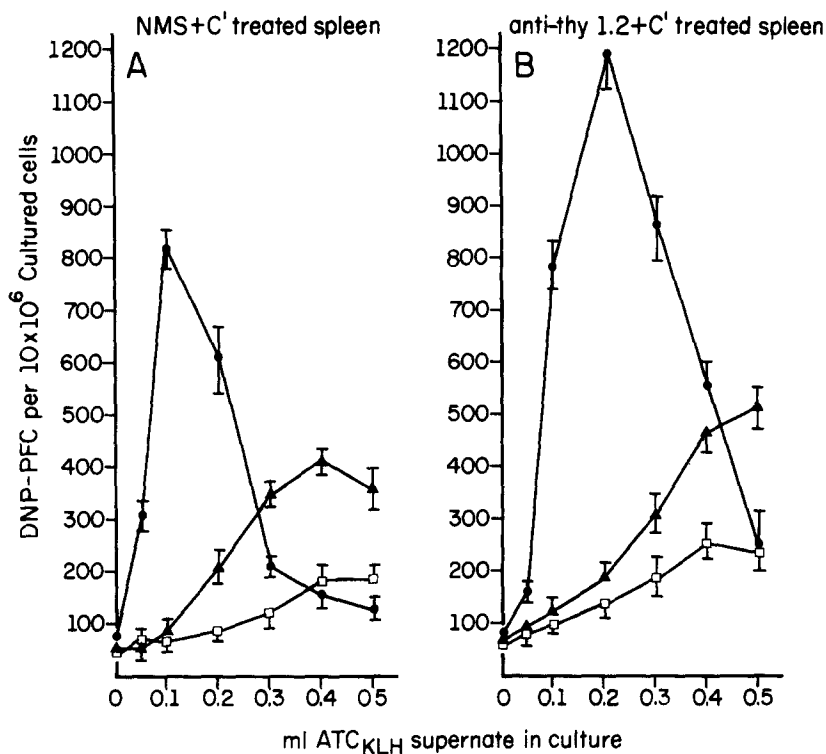
Fig. 2. Supernate stimulation of anti-DNP-PFC responses in T-cell-depleted spleen cell cultures. Supernatant culture fluid derived from T-helper cell cultures primed with KLH (ATC$_{KLH}$ S/N) was added to (A) normal mouse serum and complement-treated spleen cell cultures, or (B) anti-Thy-1.2 serum and complement-treated spleen cell cultures with DNP-KLH, (●—●); DNP-FγG, (△—△); or without hapten-carrier conjugate, (□—□). Results indicate the direct DNP-PFC response ± SD of triplicate cultures.

cultures at lower supernatant doses cannot be demonstrated in macrophage-depleted spleen cell cultures.

Absorption of Carrier-Specific Supernatant Factor with Insolubilized Carrier. Helper cell cultures generate an apparent carrier-specific supernatant activity. When assayed at an appropriate dose, supernatant culture fluid stimulates optimal enhancement of splenic anti-DNP-PFC responses to DNP conjugated to the same carrier used to prime the helper cell culture (Fig. 1). To test whether this activity has binding specificity as well as functional specificity, we absorbed supernatant fluid with carrier protein conjugated to Sepharose 4B before we assayed the supernate. The results are shown in Fig. 4. Absorption of the supernatant fluid with the appropriate carrier abolishes the carrier-specific enhancement of anti-DNP-PFC responses usually seen at the optimal supernatant dose. The nonspecific enhancement demonstrable when higher dosages of supernatant fluid are assayed is not affected by this absorption.

Absorption of Supernate with Anti-Immunoglobulin Antibodies and Alloantisera. Helper cell culture supernates were absorbed with a variety of anti-mouse immunoglobulin antibodies or the globulin fraction of alloantisera cou-
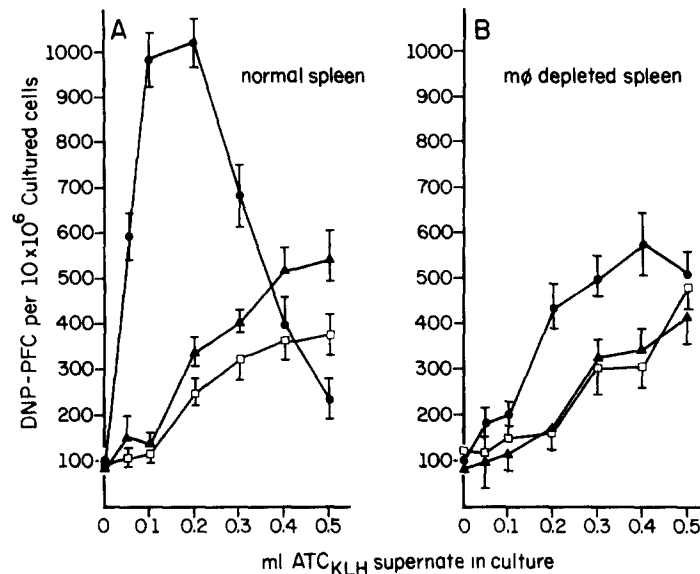
FIG. 3   Supernate enhancement of anti-DNP-PFC responses in macrophage-depleted spleen cell cultures. Supernatant culture fluid derived from T-helper cell cultures primed with KLH (ATC_{KLH} S/N) was added to (A) normal spleen cell cultures or (B) cultures depleted of macrophages with DNP-KLH, (●—●); DNP-FγG, ($\triangle$—$\triangle$); or without hapten-carrier conjugate, ($\square$—$\square$). Results indicate the direct DNP-PFC response ± SD of triplicate cultures

pled to Sepharose 4B before they were tested on spleen cell cultures. Absorption of supernatant fluid with anti-mouse immunoglobulin does not affect the stimulatory activity of such supernates on splenic anti-hapten responses. Fig. 5 shows the results of absorption of supernates from KLH-primed, thymocyte-macrophage cultures with polyvalent anti-mouse immunoglubulin antibodies (anti-$\mu,\gamma,\kappa,\lambda$-reactivity). Similar results were obtained with anti-μ-chain, anti-$\mu\kappa$-chain, and anti-γ-chain-specific antibodies.

Absorption of helper cell culture supernates with the globulin fraction of alloantisera completely removed the capacity of the supernate to stimulate carrier-specific and nonspecific enhancement of splenic anti-DNP-PFC responses (Fig. 6). In these experiments, supernates derived from KLH-primed, thymocyte-macrophage cultures were generated using either the (BALB/c × B10D2) F$_1$ hybrid (H-2^d) cells or CBA (H-2^k) cells. Before assay, each supernate was divided and absorbed with the globulin fraction of BALB/c anti-CBA sera (anti-H-2^k) or CBA anti-BALB/c sera (anti-H-2^d). Assay cultures contained hapten-primed spleen cells of the same strain from which the supernate was derived. Insolubilized alloantisera directed toward the strains from which the supernates were derived removed the stimulatory capacity of the supernates, whereas the reciprocal antisera (or preimmune sera used as the control in other experiments) had no effect on the supernatant activity.

Treatment of Supernates with Trypsin, Periodate, and Heat. Table I shows the effect of various treatment regimens on the activity of supernates. In these experiments, untreated control supernates were exposed to identical manipula-
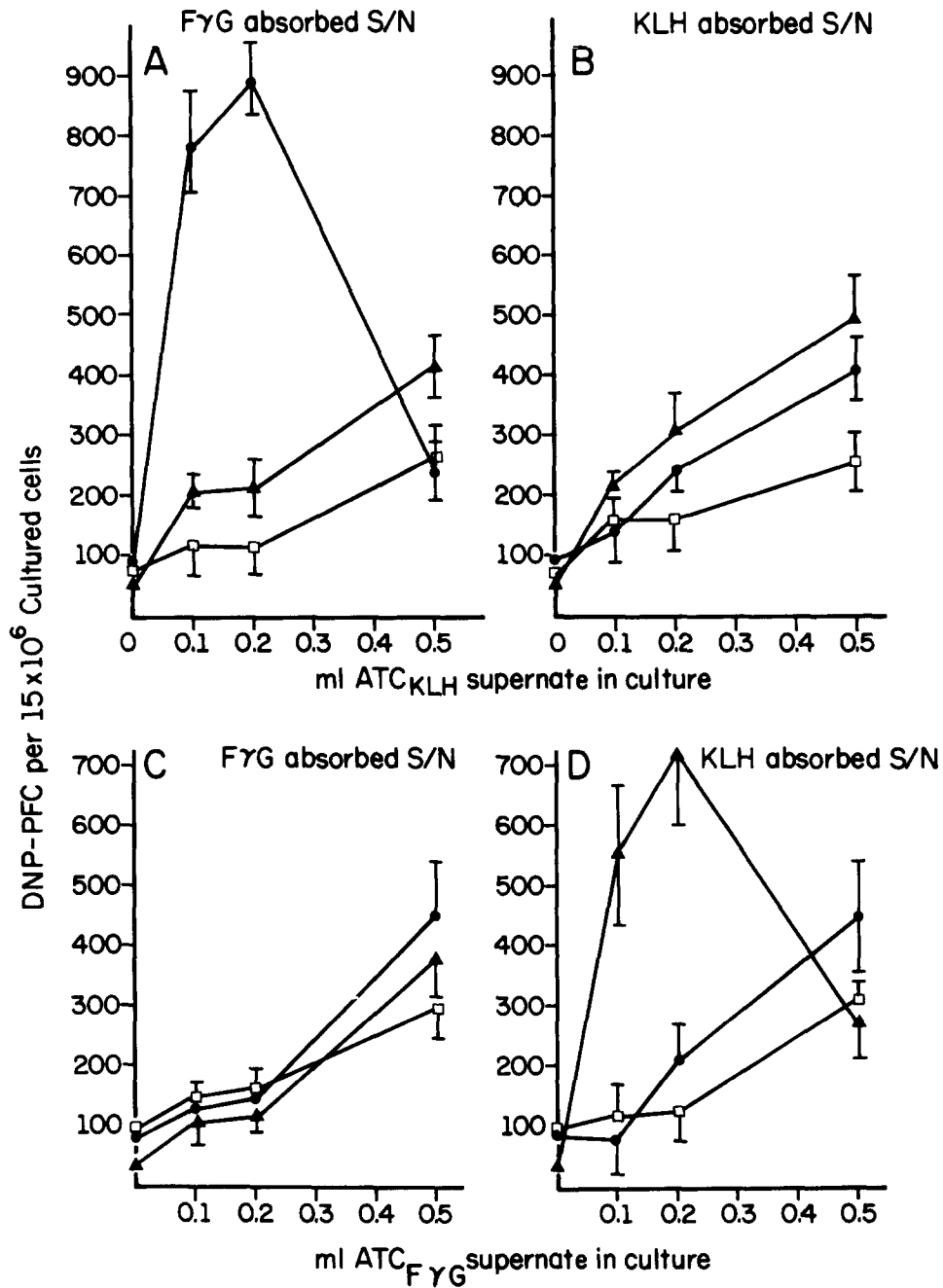
Fig 4. Absorption of carrier-specific activity with carrier. Supernates derived from thymocyte-macrophage cultures primed with KLH (charts A and B) or FγG (charts C and D) were tested at the indicated doses for enhancement of splenic anti-DNP-PFC responses after absorption with FγG (charts A and C) or KLH (charts B and D) conjugated to Sepharose 4B. Supernates were added to spleen cell cultures with DNP-KLH, (●—●), DNP-FγG, (△—△), or without additional antigen, (□—□). Results indicate the direct anti-DNP-PFC response ± SD of triplicate cultures.
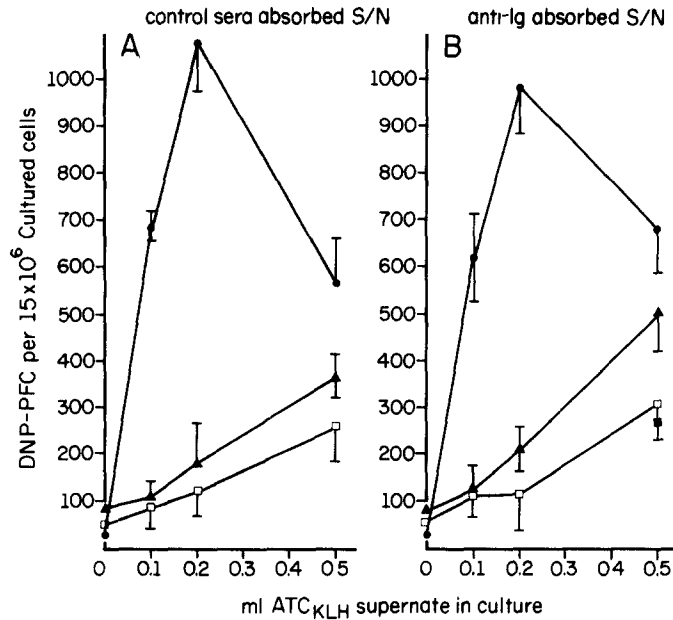
Fig. 5. Absorption of supernate with anti-mouse immunoglobulin. Supernatant culture fluid derived from T-helper cell cultures primed with KLH (ATC$_{KLH}$S/N) was tested for enhancement of splenic anti-DNP-PFC responses after absorption with (A) preimmune normal goat globulin, or (B) purified goat anti-mouse immunoglobulin conjugated to Sepharose 4B. Supernate was added at the indicated doses with DNP-KLH, (●—●); DNP-FγG, (△—△); or without additional antigen, (□—□). Results indicate the direct anti-DNP-PFC response ± SD of triplicate cultures.

tion, buffer, dilution, and dialysis conditions as the treated supernates, and both were assayed in graded doses on spleen cell cultures similar to the way experiments depicted in Figs. 4–6 were done. For simplicity, the results are tabulated as the percent inhibition of supernatant activity resulting from the treatment. This was calculated at the dose of supernate optimal for demonstrating carrier-specific stimulation as well as the dose for demonstrating nonspecific stimulation of splenic anti-DNP-PFC responses. As can be seen in Table I, trypsin treatment, periodate oxidation, and heating at 56°C for 1 h destroyed the stimulatory capacity of supernates.

To ensure that the inhibition seen was due to alterations in the active factor(s) in the supernate rather than due to the generation of inhibitory substances in the supernate by the treatment regimens, we set up a third group of cultures and added equal amounts of treated and untreated supernate to the same spleen cell cultures. Trypsin-treated and periodate-treated supernates did not interfere with the enhancing capacity of untreated supernates when both were present in the same culture. However, at low dosages, supernates that were heated at 56°C minimally depressed the stimulatory properties of untreated supernates. At high dosages (0.5 ml) the heated supernate markedly inhibited the expected response of control supernates. In addition, heated supernate markedly suppressed the response of spleen cell cultures to DNP-Ficoll and the primary anti-SRBC response without a significant effect on cell culture recovery or viability.
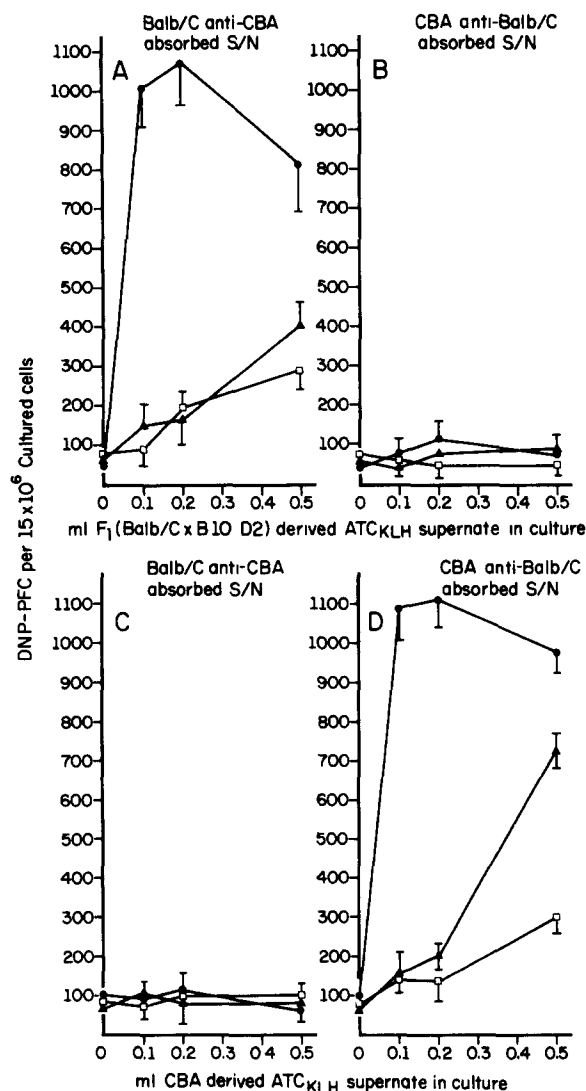
FIG. 6. Absorption of helper cell culture supernates with alloantisera. Supernates from helper cell cultures primed with KLH (ATC$_{KLH}$ S/N) were generated using (BALB/c × B10D2) F$_1$ hybrid cells (charts A and B) or CBA cells (charts C and D). Before assay in culture containing spleen cells from the same strain, supernates were absorbed with Sepharose-conjugated globulin fractions of BALB/c anti-CBA serum (charts A and C) or CBA anti-BALB/c serum (charts B and D). Cultures contained DNP-KLH, (●—●); DNP-FγG, (△—△); or no added antigen, (□—□). Results indicate the direct anti-DNP-PFC response ± SD of triplicate cultures.

Discussion

With a culture system originally described by Feldmann and coworkers (29–32), we have attempted to study T-helper cells derived from in vitro culture. In the modification of this system, purified thymocyte populations cultured with macrophages (in the absence of B cells) can be primed with soluble carrier

TABLE I

Inhibition of T-Helper Cell Culture Supernate Activity by Various Treatments

Treatment*	Percent inhibition of supernate capacity to stimulate:‡	
	Carrier-specific responses	Nonspecific responses
Trypsin	99 (96–102) (n = 3)§	99 (93–104) (n = 3)
Periodate	97 (90–105) (n = 3)	84 (67–93) (n = 3)
56°C for 1 h	94 (84–103) (n = 3)	106 (101–112) (n = 3)‖

* See text

‡ The increment (ΔRx S/N) in the anti-DNP-PFC responses in cultures with pretreated supernate above the responses in otherwise identical cultures without supernate were compared to the increment (ΔS/N) found with untreated control supernates and expressed as percent inhibition: ([ΔS/N] – |ΔRxS/N])/([ΔS/N]) × 100. These inhibitions were calculated for conditions (supernate dose, antigens) optimal for demonstrating carrier-specific and nonspecific stimulation

§ Average inhibition in percent, (range), n =number of separate experiments
‖ See text.

protein under conditions selected to generate helper cells (28). T-helper cells or their products are assayed in cultures of spleen cells from mice primed with the T-independent antigen DNP-Ficoll. This antigen would be expected to prime the spleen cells for an enhanced IgM and IgG anti-DNP-PFC response to DNP-protein conjugates under the influence of carrier-specific T-helper cells (28, 38).

The present results describe the activity of supernatant factors derived from in vitro primed T-helper cell cultures. Supernates stimulate both carrier-specific and nonspecific enhancement of splenic anti-DNP-PFC responses to DNP-carrier conjugates (Fig. 1). The carrier-specific activity can be absorbed from the supernate with insolubilized carrier protein (Fig. 4) and is further distinguished from the nonspecific activity by dosage optimum (Fig. 1) and the requirement for macrophages for full expression of its activity (Fig. 3). Both activities are sensitive to trypsin and periodate treatment (Table I) and are removed by alloantisera (Fig. 6) but not by a variety of anti-mouse immunoglobulin sera (Fig. 5). The active factors derived from this system appear to be glycoproteins antigenically related to alloantigens. In view of the number of T-cell mediators reported to play some role in the antibody response, it is of interest to compare the activity described here to factors described by others.

Despite the fact that several reliable in vivo methods exist for generating and assaying T-helper cells with a high degree of antigen specificity (1–3, 12–14), no integrated concept of the molecular events involved has emerged. This is partially attributable to the complexity involved in the generation and assay of T-helper cells. While there is ample evidence that T cells produce mediators that regulate B-cell responses (9), differential responses to mediator dose, timing, type of response generated (IgG or IgM, polyclonal or antigen specific), antigen requirements, and requirements for accessory cells contribute to the complexity and difficulty in interpreting various in vitro and in vivo systems. Both antigen-specific and nonspecific factors, which can be stimulatory or inhibitory, have been found in different systems by a number of investigators using antigen-primed or allogeneically stimulated T cells as a source of mediators (9–27).

For example, both Feldmann and Taussig and their respective co-workers have examined antigen-specific factors derived from "educated" T cells (spleen cells from irradiated mice injected with thymocytes and antigen). Assaying the factor in vitro, Feldmann et al. (11) have obtained evidence that a special class of antibody (IgT) is produced which specifically concentrates antigen at the surface of a third cell, the macrophage, and presents it as a multideterminant array to the surface of B cells. Taussig and co-workers, assaying mediators from these cells in vivo, have found an antigen-specific T-replacing factor antigenically related to products of the major histocompatibility locus, i.e., Ia antigens coded for in the K-end of the H-2 locus (13, 14). Our carrier-specific factor, while resembling Feldmann's factor in dose response (Fig. 1), antigen-binding properties (Fig. 4), and the macrophage requirement for full functional activity (Fig. 3), is not an immunoglobulin (Fig. 5), but does contain alloantigens (Fig. 6). Our factor then is an apparent in vitro assayed correlate to the factor described by Taussig and Munro.

It has been suggested that Taussig and Munros' assay system, a 12- to 14-day response in the B mouse, contains residual T cells. Also on the basis of Feldmann's data and that of other groups that have implicated a role for syngeneic macrophages or their products in the induction of T-cell responses, these alloantigen-related, antigen-specific factors may actually be interpreted as macrophage derived; they act primarily on T cells and secondarily on B cells (31).

Several lines of evidence suggest that the factors described in this communication are derived from T cells and do not require additional T-cell interaction. We have shown in the companion paper that T-helper cells are generated in vitro only with the appropriate combination of antigen, thymocytes, and macrophages, and that T-cell proliferation in vitro is required for the activation of functional T-helper cells (28). Parallel studies with supernates reveal that active supernates are generated only in cultures containing proliferating T cells. (This data alone does not rule out the generation of a macrophage factor dependent on T cells). Furthermore, supernates are proportionately more active on spleen cell cultures which have been depleted of T cells by anti-Thy-1.2 serum and complement treatment (Fig. 2). This treatment is sufficient to abrogate T-cell involvement in the primary in vitro response to SRBC and, unless the thresholds of depletion required are different, would be expected to abrogate an antigen-alloantigen induction of T cells in vitro (31). Finally, the 4-day culture period is optimal for demonstrating supernatant activity in spleen cell cultures; however, according to our data and that of Feldmann (28, 32), this 4-day period would be too short for both macrophage induction of T-helper cells and the stimulation of an optimal B-cell response to soluble antigen in vitro. While we cannot rule out a minor role for the alloantigen-related factors described here acting in the induction of T cells (helper or suppressor), a major mechanism of alloantigen-related T-cell products in this system is in the stimulation of B cells directly (nonspecific enhancement) or through adherent cells (carrier-specific enhancement) (Figs. 2 and 3).

The presence of B cells in educated T-cell preparations has particular relevance in the interpretation of data implicating immunoglobulin as the cooperative T-cell factor. It has been shown that T cells under a variety of conditions can bind cytophilic immunoglobulin (39–42), and that T-cells with cytophilic anti-

body can function to stimulate specific B-cell responses (10, 43). While there is no doubt that immunoglobulin perhaps derived from T cells can have a regulatory role (9–12, 25), Feldmann and Tada, who have described T-cell-derived regulatory factors related to the immunoglobulins, have pointed out that there is no evidence that such factors are actually synthesized by T cells (12, 25). For these reasons we have attempted to study factors derived from T cells cultured in the absence of B cells (28). The reasoning is that if an immunoglobulin factor is detected this system would readily lend itself to appropriate synthetic studies. The activity derived from this system is not, however, an immunoglobulin-related antigen.

It should be noted that our anti-immunoglobulin reagents were produced with myeloma proteins and were absorbed with thymocytes before use. Thus, although the antisera retained anti-immunoglobulin specificity, any small portion of cross-reacting determinants present in the T-cell factor would probably not be detected. Furthermore, in view of recent evidence suggesting that T-cell receptors may share idiotypic determinants with B-cell immunoglobulin (44, 45), idiotypes other than those present on the myeloma immunogen would not be detected.

Also detected in this system is a nonspecific stimulatory activity related to alloantigens. We interpret the nonspecific enhancement of PFC responses seen with higher supernatant dosages as a polyclonal activation of B cells because the stimulation is not confined to DNP-specific PFC's (46). We measured DNP-specific plaques with TNP-coated SRBC using a hapten-inhibition technique (28). Anti-TNP-SRBC PFC's inhibition by 5×10^{-4} M DNP-L-lysine are considered to be of sufficient avidity to be DNP specific (47). Using this technique, we obtain somewhat lower numbers for DNP-specific plaques in vitro than with the more conventional technique of subtracting background PFC's directed toward unconjugated SRBC. Regardless of which technique is used the number of anti-TNP-SRBC PFC's detected in the presence of hapten and the number reacting with unconjugated SRBC is increased in cultures containing greater amounts of supernate. Furthermore, at this dose of supernate, the response to an unrelated antigen such as SRBC or DNP conjugated to a carrier not used in the generation of the supernate is enhanced (Fig. 1) provided macrophages are present (Fig. 3). In this respect the factor closely resembles a factor derived from allogeneic stimulation of T cells (3, 17–22). This factor, designated allogeneic effect factor (AEF) and related to antigens coded for in the Ia region of the H-2 locus, has been extensively studied by Katz and co-workers. Although it does not display any known antigenic specificity, AEF stimulates both background PFC responses and specific responses to soluble antigen provided antigen is present with macrophages (19, 20).

Whether the alloantigens described here are related to the Ia sublocus or whether or not the specific and nonspecific factors are the same alloantigen cannot be determined as yet. The alloantisera used in this study were produced against the entire H-2 locus and probably contain other specificities as well (i.e., Ly difference). This will require alloantisera produced in congenic mouse strains with more precise specificities. If it turns out that both activities contain the same alloantigenic determinants, one could postulate a factor possessing non-

specific B-cell-activating properties which, when also possessing antigen-binding properties, preferentially concentrates this activity at the surface of antigen-specific B cells by virtue of a common receptor avidity for antigen. Such a mechanism along with the additional caveat that too much localization of the polyclonal activator at the B-cell surface results in paralysis would explain the dose responses found in this system. A similar mechanism has been proposed for the antigen-specific and nonspecific polyclonal response to DNP-lipopolysaccharide (46).

Analysis of tissue culture supernates is complicated in that the activity observed may reflect a balance between stimulatory and inhibitory activities. Moreover, helper factors with stimulatory or inhibitory properties may exhibit dosage and timing optimums depending on assay conditions (3, 4, 9, 10, 21–25). The culture conditions used here were selected to maximize the generation of a stimulatory helper T cell, and the supernatant factors derived presumably reflect a balance of activities favoring the demonstration of stimulation. Ishizaka and Adachi (8) described a culture system which is essentially similar to ours except that T-cell-enriched spleen cells and higher antigen doses are used. In their system, specific helper or suppressor cells can be generated depending on the presence or absence of macrophages. Alloantigen-related factors have been implicated in nonspecific and antigen-specific suppression as well as in stimulation (3, 13, 14, 17–21, 23, 26, 27). It is unclear as yet whether these alloantigens are distinctly different molecules or whether the opposing activities observed reflect the activity of identical antigens with potentially different pathways of cellular interaction, quantitatively regulated target cell sensitivity, qualitative conformational differences determining the activity observed, or associated molecular structures with different activities. It is of interest, in this respect, that heat treatment of helper cell culture supernates in our system not only destroyed its stimulatory capacity, but resulted in a markedly suppressive activity. This activity is nonspecific with respect to antigen response and not explained by inhibition of cell culture recovery or viability. Preliminary experiments indicate that the suppressive activity is also removed by alloantisera. Whether this represents the same alloantigen conformationally altered or aggregated by heat to result in suppressor activity must be determined by more definitive identification of the alloantigens involved.

Summary

Supernates derived from in vitro generated T-helper cells have been analyzed for their capacity to substitute for T-cell carrier reactivity. T-helper cell supernates stimulate both a carrier-specific and nonspecific anti-DNP-PFC response to DNP-carrier conjugates in cultures of hapten-primed spleen cells. The carrier-specific and nonspecific activity can be distinguished by dosage optimum, antigen requirements, binding specificity for carrier, and in the requirement for additional splenic adherent accessory cell involvement. The active factors produced in this system are heat labile and sensitive to trypsin and periodate. They are removed by absorption with alloantisera directed toward the strain from which the supernate was derived but not by a variety of anti-immunoglobulin sera.

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