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T Cell-dependent Mediator in the Immune Response

II. PHYSICAL AND BIOLOGICAL PROPERTIES

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Summary. Supernatants were obtained from cultures of keyhole limpet haemocyanin (KLH) primed mouse spleen cells, and also from cultures of KLH-educated, thymus-derived lymphocytes (T cells) after re-exposure of cells to the original priming antigen, KLH.

These supernatants were able to facilitate the immune response of bone marrow-derived lymphocytes (B cells) to sheep erythrocytes (SRBC).

Certain physical and biological properties of such supernatant activities are described.

INTRODUCTION

The mechanism by which thymus-derived lymphocytes (T cells) facilitate the response of bone marrow-derived lymphocytes (B cells) has been the subject of intensive investigation in recent years, particularly in *in vitro* culture systems which are thought to simulate many components of the *in vivo* immune response. It has been shown in many such *in vitro* systems that both specific (Feldmann and Basten, 1972a; Yu and Gordon, 1973) and non-specific factors (Dutton, Falkoff, Hirst Hoffman Kappler, Kettman, Lesley and Vann, 1971; Schimpl and Wecker, 1972; Rubin and Coons, 1972; Britton, 1972; Maillard and Bloom, 1972; Doria, Agarossi and Di Pietro, 1972; Gorczynski, Miller and Phillips, 1973a; Waldmann and Munro, 1973) can be elicited from T cell-containing cultures which can partially replace T-cell helper activity. In this communication we provide data concerning the mechanisms of production and of action, and certain physical characteristics of a T cell-dependent factor produced by the interaction of specific T cells and antigen.

MATERIALS AND METHODS

Mice of the CBA/Ca strain were used throughout.

Thymectomy was performed by the method of Miller (1960). Approximately 4 weeks later such mice were irradiated with 875 rads from a ^{60}Co source and approximately 4×10^6 syngeneic bone marrow cells intravenously. Terramycin (Pfizer) was routinely added to the drinking water.

The spleens of the thymectomized and bone marrow-reconstituted mice were used 5–12 weeks later as a source of 'B cells' *in vitro*.

Antigens

Sheep erythrocytes (SRBC) were from one donor (X64) and were stored in Alsever's solution for up to 3 weeks before use.

Keyhole limpet haemocyanin (KLH) was obtained from Calbiochem.

Immunization

Immunization for the production of KLH-educated T cells was carried out by intraperitoneal (i.p.) injection of 500 μg of KLH (without added adjuvant) into mice which had been irradiated with 875 rads, and had subsequently received 10^8 syngeneic thymocytes intravenously.

Priming of normal mice was performed by the i.p. injection of 200 μg KLH in Freund's complete adjuvant, followed 4 weeks later by 50 μg KLH i.p. After 5 weeks the spleen cells of these mice were used to produce active supernatants.

Culture conditions

Spleen cells for culture were prepared as previously described (Waldmann, Munro and Hunter, 1973).

The culture medium used was RPMI 1640 to which was added supplements as described by Mishell and Dutton (1967), and also nucleosides and 5×10^{-5}M 2-mercaptoethanol as suggested by Click, Benk and Alter (1972).

'Adherent' cells were obtained from the spleens of 'B mice' by culturing 6×10^6 spleen cells/ml for 1 hour at 37°, and then washing off non-adherent cells (Mosier, 1967).

The system used to demonstrate and analyse 'supernatant' activity was based on that developed by Dutton (personal communication) in which 6×10^5–1×10^6 B cells were cultured in the wells of Cooke's microtitre trays in a final volume of 110 μl of medium. Ten microlitres of this total volume comprised an additional 1 per cent SRBC as antigen.

The plaque-forming cell (PFC) response was assayed, except where indicated, 4 days after the initiation of culture.

Plaque-forming cell assay

PFC were determined by a modified Jerne haemolytic plaque assay. 100 μl of 5 per cent SRBC in Eagle's HEPES were added to each culture pot and subsequently mixed rapidly with 0·25 ml of 0·6 per cent agarose, and the mixture poured onto agarose-coated slides. The slides were then incubated for 1½ hours after which guinea-pig serum was added.

Sepharose immunoabsorbent

Twenty milligrams of a 33 per cent ammonium sulphate precipitate of a rabbit anti-KLH serum were coupled to 1 gram of cyanogen bromide-activated Sepharose (Pharmacia). Twenty milligrams of normal rabbit IgG was similarly coupled to control columns.

Before use the columns were washed thoroughly with Eagle's HEPES. Culture medium supernatant preparations were then run through such columns in 2-ml volumes.

Anti-brain θ serum

Rabbit anti-mouse brain θ antiserum was prepared as described by Golub (1971) and absorbed with mouse erythrocytes and acetone-dried mouse liver powder before use. Guinea-pig serum was absorbed twice with 1/10th volumes agarose before use as a source

of complement for the depletion of T cells by cytolysis with anti-θ serum. 100×10^6/ml spleen cells were incubated with a 1:4 dilution of the serum in Eagle's HEPES for 30 minutes in the cold. Subsequently the mixture was diluted in 10 ml of Eagle's HEPES, the cells spun down and resuspended at 10^8/ml in a 1:8 dilution of guinea-pig serum, and left for 30 minutes at 37°, washed and resuspended in the required volume of culture medium.

Sephadex G-200 chromatography

Molecular weight estimations of supernatant activities were made after fractionating concentrates of active supernatants through Sephadex G-200 columns which had been previously equilibrated with culture medium lacking bicarbonate. Two-millilitre fractions were stored at −20°, and later tested for their ability to reconstitute B-cell responsiveness to SRBC.

TABLE 1

THE EFFECT OF VARIOUS CULTURE SUPERNATANTS PREPARED ON THREE OCCASIONS ON B-CELL RESPONSIVENESS TO SRBC

Preparation of supernatant	PFC		
	Experiment 1	Experiment 2	Experiment 3
Medium control + KLH	1	4	2
Adherent cells + KLH	7	7	8
Educated T cells + KLH	25*	16*	6
Adherent cells + educated T cells + KLH	90*	95*	122*

* Significant enhancement above control responses. $P < 0{\cdot}05$.

10^7 nucleated spleen cells from KLH-educated mice were cultured for 36 hours with 30 μg of KLH, with or without adherent cells from B mice. Supernatants were taken and tested at a concentration of 1:1 with medium for their ability to reconstitute B-cell responsiveness to SRBC in microculture (see Materials and Methods section). Results are expressed as PFC/microculture. Each value represents the geometric mean of eight replicate cultures.

Appropriate molecular weight markers were later passed through the same columns.

Statistics

Log-transformed data were subjected to an analysis of variance and Duncan's multiple range testing on IBM 370 at the Computer Laboratory, University of Cambridge.

Significant differences between groups were assessed at the 5 per cent level only.

RESULTS

SUPERNATANTS FROM CULTURES OF EDUCATED T CELLS AND ANTIGEN

Supernatants from cultures of educated T cells can facilitate the response of B cells to SRBC in vitro

Supernatants were obtained from cultures of KLH-educated T cells by incubating the cells derived from one spleen equivalent (approximately 10^7 total nucleated cells) with 5–30 μg KLH for 40 hours in a 1-ml culture volume.

Table 1 demonstrates that such supernatants had a small degree of facilitatory activity in experiments 1 and 2, but none in experiment 3. However, in all cases the addition of adherent cells from B mice to cultures used in preparing the supernatants produced marked increases in their facilitatory activity.

Subsequent analysis of this activity showed it to be non-dialysable and stable on storage at both −180° and −20°. Supernatants prepared by incubation of educated T cells and adherent cells are designated Tm in the paper.

Requirement for antigen

Although KLH was required to elicit the production of factor by cultures containing KLH-educated T cells (Waldmann *et al.*, 1973) it seems that the biological activity of the factor was not associated with nor required further KLH for its expression. Table 2 demonstrates this fact.

Supernatants from cultures of KLH-educated T cells and adherent cells incubated with 3 μg KLH/ml (Tm) were prepared. Two-millilitre quantities of supernatants were passed through 8-ml volume Sepharose immunoabsorbent columns as described in the Materials and Methods section. The effluent material was subsequently millipored and stored at −20° until testing. It can be seen that no activity was lost after treatment with immunoabsorbents.

The test immunoabsorbent was subsequently shown to be able to bind at least 5 μg of ^{125}I-labelled KLH from 2 ml of solution.

TABLE 2

THE EFFECT OF PASSING TM SUPERNATANTS DOWN AN ANTI-KLH IMMUNOABSORBENT

Treatment of supernatant	PFC/culture
Untreated	34*
Sepharose anti-KLH immunoabsorbent	35*
Passed through normal rabbit IgG Sepharose immunoabsorbent	25*
Normal medium	6

* Significant enhancement above medium control. $P < 0{\cdot}05$. These three values were not significantly different from each other.

Tm supernatant was made with 3 μg KLH/ml. This was passed through anti-KLH-coated Sepharose and control normal rabbit IgG-coated Sepharose. Effluent activity was tested in microculture for ability to facilitate B-cell responsiveness to SRBC. Results are expressed as PFC/microculture. Each value represents the geometric mean of six replicate cultures.

Molecular weight

Fig. 1 demonstrates the results of testing fractions of Tm supernatant from a G-200 column for their ability to facilitate the response of B cells to SRBC.

Maximum significant biological activity was detectable in fractions eluting in the molecular weight range of 30,000–50,000.

SUPERNATANTS FROM KLH-PRIMED NORMAL SPLEEN CELLS

Spleen cells from mice primed with KLH as described in the Materials and Methods section produced supernatants after incubation with 10 μg KLH/ml with a similar facilitatory effect on B-cell responsiveness to SRBC.

Titration of supernatant activity

Fig. 2 demonstrates the titration curve for such a supernatant (Ss), and shows that enhancing activity is lost at a quite low dilution of 1:8.

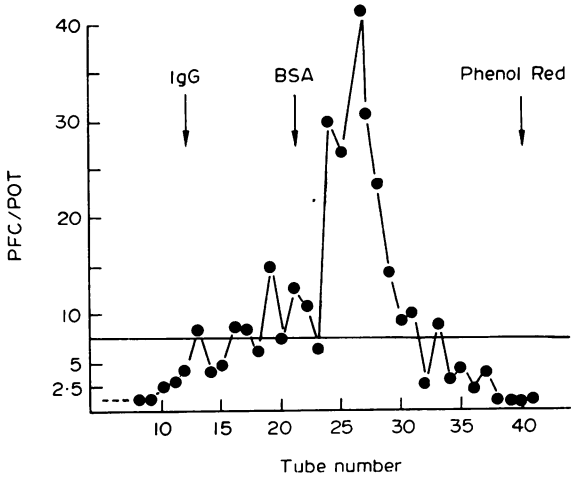

FIG. 1. Fractionation of Tm supernatant on Sephadex G-200. The supernatant was made with 10 μg KLH. A 2-ml concentrate of 16 ml of starting Tm supernatant was loaded onto the column (see Materials and Methods section). Fractions were tested for their ability to facilitate B-cell responsiveness to SRBC in microculture. Results are expressed as PFC/microculture. Each value represents the geometric mean of six replicate cultures. The line overlying values not significantly different from background controls is shown ($P < 0{\cdot}05$).

Molecular weight

A concentrate of Ss supernatant was fractionated on a Sephadex G-200 column as before. The column was, however, on this occasion, equilibrated with medium lacking bicarbonate and FCS, but containing 0·1 per cent BSA.

Fig. 3 demonstrates that Ss contains activity with a similar chromatographic profile with a peak, and maximum significant activity occurring between approximately within the 25,000–60,000 molecular weight range.

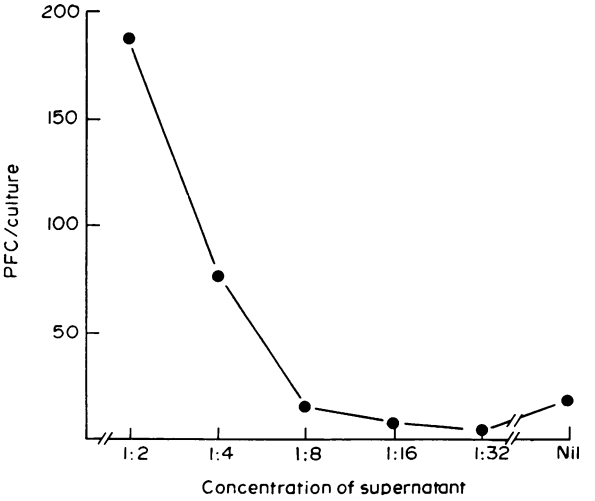

FIG. 2. The titration of Ss supernatant activity on B-cell responsiveness to SRBC. Each value is the geometric mean of eight replicate microcultures. Results are expressed as PFC/microculture. Significant enhancement was seen at dilutions of 1:2 and 1:4 only compared to medium controls. $P < 0{\cdot}05$.

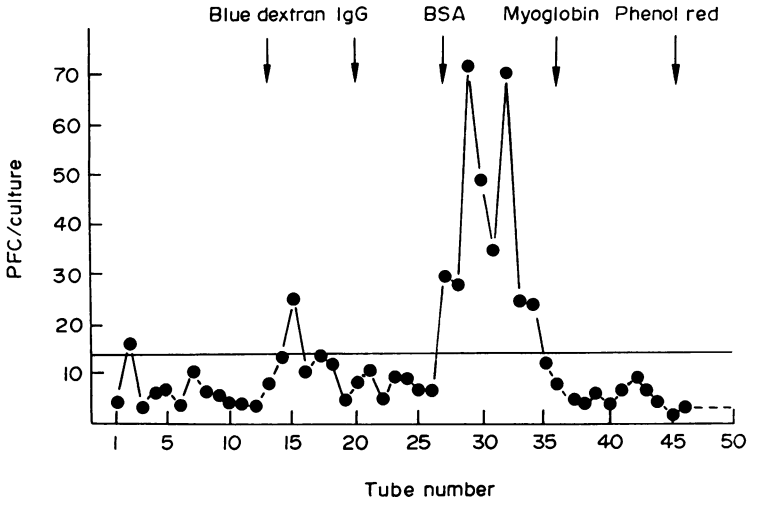

FIG. 3. Fractionation of Ss on Sephadex G-200. A 1-ml concentrate of 6 ml of starting material was fractionated on Sephadex G-200. Fractions were tested as described in Fig. 1. Each value represents the geometric means of six microcultures. Results are expressed as PFC/culture. A line overlying values not significantly different from background controls is shown ($P<0{\cdot}05$).

Ss activity was found to be heat-stable to treatment at 56° for 30 minutes, non-dialysable and not inhibited by 10^{-3} M paramethyl sulphonyl fluoride (PMSF), a protease inhibitor. Absorption of Ss with 1:5 volumes of washed SRBC did not diminish its activity.

The ability of KLH-primed spleen cells to non-specifically enhance culture responses to SRBC is abolished by anti-θ treatment, and can be restored by KLH-educated T cells (unpublished data). This in relation to the previous data suggests that it is likely that Tm

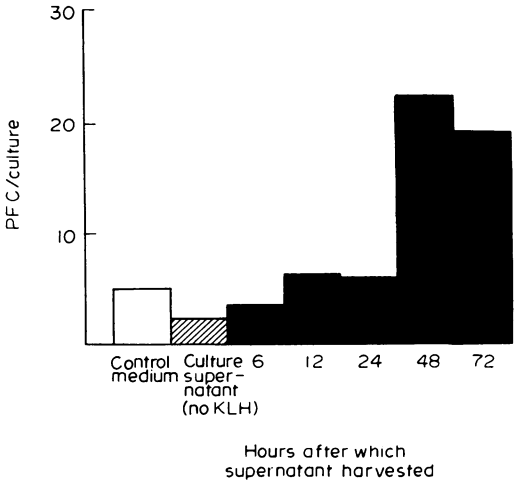

FIG. 4. Time course for the appearance of detectable Ss in cultures. 1×10^7 KLH primed cells were cultured for varying periods with 10 μg of KLH, and supernatants taken, stored at −20° till tested for facilitation of B-cell responsiveness to SRBC. Each value represents the geometric means of eight replicate cultures. Significant enhancement above from medium controls seen at 48 hours and 72 hours ($P<0{\cdot}05$).

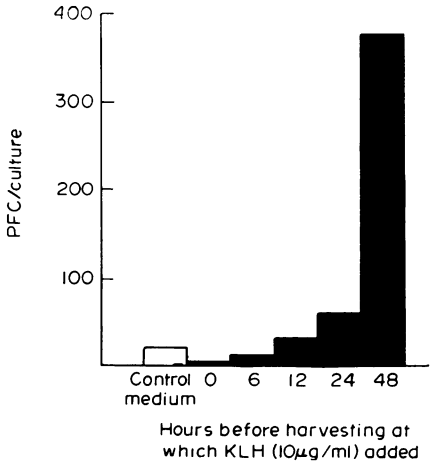

FIG. 5. The effect of adding KLH at various intervals to cultures of KLH-primed cells on subsequent activity of the Ss supernatant. The supernatant was assayed for ability to facilitate B-cell responsiveness to SRBC. Significant enhancement above medium control was seen at 12, 24 and 48 hours ($P < 0{\cdot}05$). Each value represents the geometric mean of eight replicate cultures.

and Ss supernatants contain identical active moieties. The greater magnitude of biological activity of Ss, and its ease of production, rendered it more suitable for further analysis. In fact Ss could be produced and exert its effect in medium containing foetal calf serum, rabbit serum, human serum and serum-free medium.

BIOLOGICAL ACTIVITY

The optimal facilitatory capacity of Ss was obtained only after KLH-primed cells had been incubated with KLH for 48 hours. This can be seen in Figs 4 and 5 where KLH was either added at the initiation of cultures which were then harvested at varying times, or where KLH was added at varying intervals and cultures all incubated for similar periods

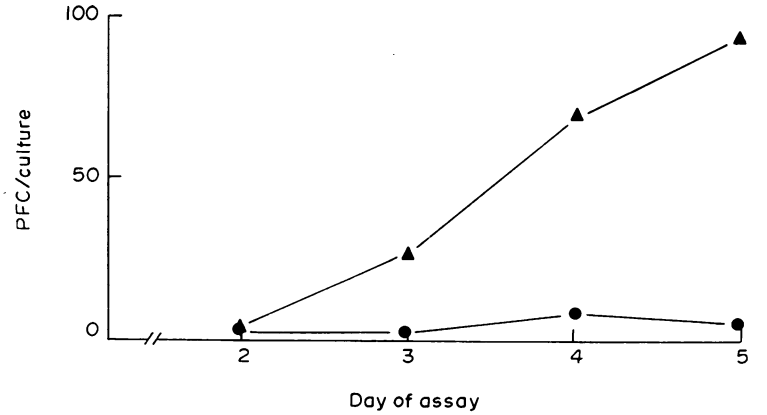

FIG. 6. The ability of Ss supernatant to facilitate responsiveness of anti-θ-treated B-cells to SRBC. B cells were treated with anti-B θ as described in the Materials and Methods section. Ss supernatant was tested at a concentration of 1:1 with medium. (▲) Ss supernatant. (●) Control medium. Each value represents the geometric mean of eight replicate cultures. Significant enhancement was seen on days 3, 4 and 5 ($P < 0{\cdot}05$) compared to medium controls assayed on those days.

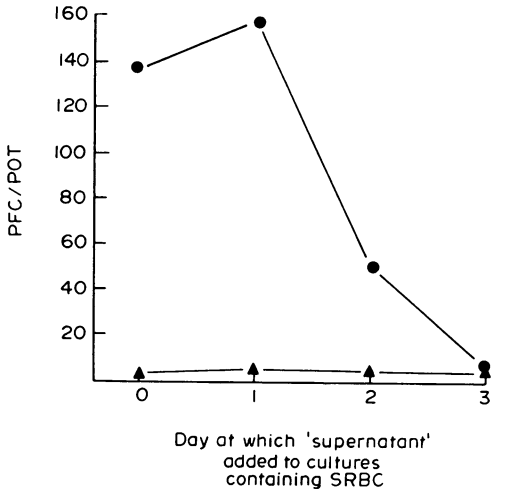

FIG. 7. The effect of adding Ss to microcultures of B cells at various times in the culture period, on the subsequent response to SRBC. B cells were plated out in 100-μl quantities. Supernatants or control medium were added by replacing 50 μl of culture medium with test material.

Each value represents the geometric mean of eight replicate microcultures. (●) Ss supernatant. (▲) Medium. Significant enhancement was seen on days 1 and 2. There was no significant difference in the degree of enhancement if supernatant was added on day 0 or day 1, but the response was significantly lower to these if Ss was added on day 2. POT = optimal PFC response. ($P<0{\cdot}05$.)

before harvesting. The latter control was performed to exclude the possibility of a requirement for some conditioning factor for optimal production of biological activity.

Ss was able to facilitate responsiveness of B spleen cells which had been further treated with anti-θ and guinea-pig complement (Fig. 6). Thus it seems highly unlikely that this material needs T cells specific for SRBC to have an effect on B-cell responsiveness. T cell-derived supernatant activity (Tm) behaves similarly.

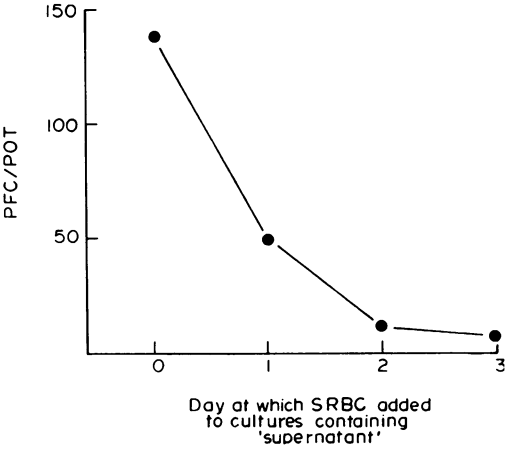

FIG. 8. The requirement for SRBC early in the culture period for optimal enhancement by Ss supernatant. Ss was added to microcultures of B cells at 1 : 1 dilution. Ten microlitres of SRBC were added at daily intervals and the subsequent PFC response was assessed. Results are expressed as PFC/microculture. Each value represents the geometric mean of eight replicate microcultures. Significant enhancement was seen if SRBC were present on day 0 or day 1. However, the effect on day 0 was higher than day 1. POT = optimal PFC response. ($P<0{\cdot}05$.)

Ss produced optimal PFC responses when introduced into cultures on either day 0 or day 1 of the 4 day culture (Fig. 7), which was also true for Tm supernatant. The data in Fig. 8 show that an early requirement for SRBC was required for optimal responsiveness to occur with Ss supernatant. It would seem, therefore, that the active moiety in Ss needs to act early on in the *in vitro* immune response to SRBC, and that it is unlikely to be solely a 'differentiating' factor, nor its effect solely a consequence of some mitogenic activity on B cells, as synergism with antigen (SRBC) was required early in the response.

DISCUSSION

These experiments show that, within a syngeneic system, T cells when activated by their specific antigen can initiate the production of mediators which can facilitate the response of B cells to a non-cross-reacting antigen SRBC. The mediator was neither physically associated with its inducing antigen nor absorbed out by absorbing supernatants with SRBC. This data confirms that described by others in both syngeneic and allogeneic situations. The production of such an antigen induced non-specific factor (NSF) is enhanced by the addition of adherent cells, and it appears in preliminary experiments, that NSF once produced can facilitate B-cell responsiveness to SRBC after carbonyl-iron-binding cells have been depleted from this population (unpublished data). Three possibilities seem likely for the facilitatory activity of adherent cells: (i) the requirement for some conditioning factor provided by adherent cells to render cultures suitable for T-cell activation; (ii) a requirement for macrophages to provide appropriate presentation of antigen (KLH) to T cells; (iii) a direct role of adherent macrophages in the production of NSF.

It seems likely that the active moiety acts directly on B cells, rather than indirectly through the activation of some serum factor, since these effects can be exerted in serum-free medium. This would seem to exclude the hypothesis that activation of the third component of complement provides the mechanism by which activation of B cells by T cell-derived non-specific factors occurs, an hypothesis recently proposed by Dukor and Hartmann (1973) unless C3 is synthesized *in vitro*.

The evidence is compelling that the NSF acts directly on B cells, and not by 'expanding' or activating a residual population of T cells specific to SRBC.

The arguments for this are twofold: (i) anti-θ-treated 'B spleen' cells respond well to SRBC in the presence of active supernatants; (ii) preliminary limiting dilution studies carried out in collaboration with Dr I. Lefkovits and Dr J. Quintans in cultures of nude mouse spleen cells indicate that NSF effectively activates nearly all available precursor B cells into clonal proliferation and antibody production, in a microculture system where specific T cells should be extremely limiting. Similar arguments to this have been made by Hunter and Kettman (1973) in their analysis of the activity of allogeneic factor. However, in contrast to this Gorczynski, Miller and Phillips (1973b) have reported different results with 'allogeneic' factor. They found that 'allogeneic supernatants' required the presence of some specific T-cell activity before B-cell responses to SRBC could be initiated. The reasons for discrepancies between groups is not clear but it would seem that minutiae of the culture system (e.g. batches of foetal calf sera) may be extremely relevant in determining what particular facet the observers attention may be focused in analysing the behaviour of these supernatants.

Molecular weight estimations of active moieties in allogeneic and this syngeneic supernatant are also disparate: Feldmann and Basten (1972b) report that allogeneic

activity was dialysable; Gorczynski *et al.* (1973a) report that allogeneic factor has a molecular weight of approximately 150,000; Hunter and Kettman (1973) report at least two activities in allogeneic supernatants with molecular weights of approximately 27,000 and a dialysable moiety. The syngeneic NSF described here moves with an apparent molecular weight of 25,000–60,000. It thus remains possible that allogeneic factor and NSF are different, and that indeed allogeneic supernatants may contain more than one helper moiety.

The question arises as to which stage in the development of a resting B cell into its progeny plasma cells NSF functions. The balance of evidence from our experiments would suggest that a large part of the activity is required early on in the SRBC response, and probably concerned with the initiation of B-cell response to this antigen.

(1) NSF added on day 0 or day 1 produce much larger PFC responses after 4 days of culture than if added on days 2 or 3.

(2) Other potential initiating sources have been systematically eliminated and yet NSF still exerted its facilitatory property on B cells. These included: (i) depletion of any residual T cells in 'B mice' by anti-θ treatment, and dilution of any specific T cells in nude mice by limiting dilution (as previously described); (ii) culture of B spleen cells in serum free medium (unpublished data) where any possible 'mitogen' of foetal calf serum is eliminated.

The only other 'initiating' factor that could not be eliminated was the antigen SRBC itself. It is known (Shortman and Palmer, 1971) that solubilization of SRBC can expose a degree of thymus independence for this complex antigen.

In contrast to this, Schimpl & Wecker (1972) reported that the allogeneic T-cell replacing factor (TRF) was most effective if added on day 2 of a 4-day culture rather than day 0. Indeed, these authors have compared our supernatant Tm with their allogeneic supernatant, and confirm that in nude mice Tm produced an optimal effect if added on day 0, and allogeneic supernatant if added on day 2 (personal communication). In addition to this Askonas, Schimpl & Wecker (submitted for publication) report that their allogeneic factor has a major component of its function the provision of a differentiation signal to B cells favouring immunoglobulin synthesis.

It would therefore seem that these two activities, NSF and TRF, are likely to be different.

It is now becoming clear that the *in vitro* immune response to sheep erythrocytes as a thymus-dependent antigen is not a completely satisfactory criterion on which to evaluate and generalize about T-cell function in the humoral immune response. The erythrocyte may have special qualities as a carrier of antigenic determinants, as well as the thymus-independent component previously discussed. We have found that a variety of non-specific agents can enhance the response of B cells to hapten (TNP) bound to erythrocytes but not to hapten conjugated to soluble thymus-dependent protein carriers in both primary and secondary challenge *in vitro* (in preparation). These non-specific agents include polymerized flagellin, endotoxin, allogeneic cells and KLH-educated T cells. Our interpretation of this data is that the erythrocyte already presents latticed antigen determinants which are just insufficient in themselves to initiate B-cell induction, but need some further activating influence to provide the final trigger. We suggest that experimentally such an activating effect can be non-specific. An extension of this argument is that T-cell specific factors are not an obligatory requirement for the initiation of B-cell activity in all thymus-dependent responses, but that there is a spectrum of thymus dependency with relatively

well latticed antigen capable of deriving their decisive triggering signals from T cell-dependent, non-specific factors, and poorly latticed antigens requiring the establishment of some degree of latticing by specific T-cell factors before triggering can take place.

The relationship between T cells mediating the specific helper effects and those initiating NSF production is not clear. However, NSF is produced optimally under conditions in which a specific suppression phenomenon mediated by T cells recognizing their specific antigen is occurring, either as a condition of a high antigen concentration or of an excess of specific T cells (Waldmann and Munro, submitted for publication). This observation, linked with that of Humphrey and Turk (1963) and also of Sterzl (1968) that unrelated delayed hypersensitivity may be associated with enhanced antibody responses to bystander antigens *in vivo*, would tend to link the production of this particular lymphokine, NSF, to a T-cell type which mediate suppression and delayed hypersensitivity. An alternative explanation is that the three phenomena of T cell-mediated suppression, specific co-operation, and production of NSF may all be a sequel to the release of specific T-cell factors. Thus specific co-operation and T cell-mediated suppression would be envisaged as representing a balance between the amount of T cell specific factor–antigen 'complexes' bound to macrophages, and that in solution free to tolerize B cells or T cells as suggested by Feldmann and Nossal (1973). On this model the production of NSF would be considered to be a consequence of T-cell specific factor–antigen complexes binding to macrophages, and in turn activating these to produce the material. This would be compatible with the finding that adherent cells were required to allow optimal production of NSF.

The relevance of NSF to the *in vivo* immune response has yet to be determined, as has even the demonstration of its production in *vivo*. Certainly, it may contribute to the effects described by a number of workers with models showing apparent non-specific enhancement or 'promotion' *in vivo* (Freund, 1951; Good, Condie, Thompson & Jensen, 1957; Humphrey & Turk, 1963; Katz, Paul, Goidl & Benacerraf, 1971; Sterzl, 1968; Mond, Takahashi and Thorbecke, 1972; Rajewsky, Roelants and Askonas, 1972).

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