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# T cell help mechanisms in the *in vitro* antibody response: the role of linked and non-linked recognition interactions

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**Summary.** Mechanisms by which T and B lymphocytes co-operate in the *in vitro* secondary antibody response to trinitrophenyl (TNP)-conjugated soluble protein antigens were investigated. The generation of antibody responses was analyzed when haptenic and carrier determinants were either linked or non-linked. Ability to co-operate through each of these mechanisms was influenced by the experimental conditions employed, particularly the mode of preparation of the T cells and the antigen concentration used. Nylon wool filtration of T cells may deplete a T helper population involved in non-linked recognition interactions. High antigen concentrations favour the non-linked form of interaction whereas low antigen concentrations favour linked recognition interaction. These data suggest that at least two distinct co-operative mechanisms co-exist. However, experimental conditions can be defined under which either one mechanism predominates or where more than one mechanism is relevant.

Abbreviations: FGG, fowl gamma-globulin; KLH, keyhole limpet haemocyanin; MHC, major histocompatibility complex; OVA, ovalbumin; PFC, plaque-forming cell; TNP, trinitrophenyl; TRF, T cell-replacing factor.

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## INTRODUCTION

It has been recognized for some years that the antibody response requires co-operation between T and B cells. However, the mechanisms by which such co-operation takes place remain controversial. A number of mechanisms have been postulated based on extensive studies with hapten-carrier systems. Under the appropriate conditions, T cells recognizing carrier determinants help B cells respond to haptenic determinants. In some instances the haptenic and carrier determinants must be covalently linked for T–B co-operation to occur; this is termed linked recognition interaction (Mitchison, 1971). In other studies no such requirement for physical linkage of the relevant determinants is seen; T cells responding to one antigen help B cells respond to determinants on a second, separate molecule. This is termed non-linked recognition interaction. T cell-derived non-antigen-specific factors, such as T cell-replacing factor (TRF), are thought responsible for help in such non-linked recognition interactions (Schimpl & Wecker, 1973; Waldmann & Munro, 1973).

There is ample evidence supporting the existence of each of these co-operative methods. However, major questions concerning antibody responses remain unresolved. For example, it is unclear to what extent these mechanisms co-exist and synergize and how the nature of the antigenic stimulus and the differentiation status of both T and B cells influence the co-operative event.

It seems likely that our limited understanding of these basic variables of cell interaction contributes to current controversies concerning the nature of major histocompatibility complex (MHC) and idiotypically related interactions in the immune system.

These questions have been extensively investigated in *in vitro* systems. Some studies have emphasized linked recognition interactions as the major mode of co-operation (Henry, 1975; Waldmann, 1975; Harwell, Kappler & Marrack, 1976) whilst others have suggested a major contribution from non-linked mechanisms (Kishimoto & Ishizaka, 1973; Hunig, Schimpl & Wecker, 1977; North, Kemshead & Askonas, 1977; Tada *et al.*, 1978; Takatsu, Tominaga & Hamaoka, 1980). A number of authors have suggested that such discrepant results stem from differences in the B cell populations. Highly activated B cells may respond well to soluble factors (and so be helped through non-linked recognition interactions) whereas resting B cells require further signals from T cells (that is, through linked recognition interactions). The experiments reported here demonstrate that T cell heterogeneity also influences the efficiency of these two mechanisms.

In this paper we describe experimental systems in which both linked and non-linked recognition interactions contribute to induction of secondary antibody responses against soluble TNP-conjugated proteins in cultures of mouse spleen cells. TNP-protein conjugates were used as immunogens and the efficiency of co-operation was compared when the relevant antigenic determinants were on the same and on separate molecules. Two factors were shown to influence the mode of interaction substantially. The first was the T cell source and the second the antigen concentration.

These studies have allowed us to define experimental conditions in which to analyse the cellular basis of H-2 restricted interactions in antibody formation. These data will be the subject of further papers.

## MATERIALS AND METHODS

*Animals*

Male and female CBA mice, 8–30 weeks old, were used. All mice were bred in the Department of Pathology, University of Cambridge.

*Antigens*

Keyhole limpet haemocyanin (KLH) and trinitrophenylated-KLH ($TNP_{1000}$KLH, asuming a molecular weight for KLH of $8 \times 10^6$) were gifts of Dr M. Baltz and Dr M. Rittenberg. Ovalbumin (OVA) and fowl gamma-globulin (FGG) were obtained from Sigma. TNP-OVA and TNP-FGG were prepared using a modification of the method of Rittenberg & Amkraut (1966).

*Immunizations*

Mice were immunized by intraperitoneal injection of 500 $\mu$g of KLH, $TNP_3$-OVA or $TNP_6$-FGG emulsified in Freund's complete adjuvant (Difco). Four weeks later all animals received an i.p. booster injection of 100 $\mu$g soluble protein in saline. KLH-primed spleen cells were taken from animals boosted 3–7 days earlier. TNP-primed B cells were taken 7–21 days after boosting.

In vivo *'education' of thymocytes*

Thymocytes were 'educated' by exposure to KLH in irradiated, syngeneic recipients. Mice aged 12 or more weeks received 750 rads from an X-ray source. Four to 14 hr later these animals were injected i.v. with $10^8$ thymocytes and i.p. with 100 $\mu$g of soluble KLH in saline. Seven days later, spleens from these animals were used as a source of KLH-specific T helper cells.

*Preparation of cell suspensions*

*T cells.* In some experiments spleen cells from KLH-primed animals or 'educated' thymocytes were treated with mitomycin C (25 $\mu$g/ml/$5 \times 10^7$ cells for 20 min at 37°) and then used as a source of T helper cells without further purification. Alternatively, T cells from KLH-primed animals were enriched by passage through nylon-wool columns (Julius, Simpson & Herzenberg, 1973) and the non-adherent cells were then treated with mitomycin C (detailed above). In all cell preparations, erythrocytes were lysed by treatment with an ammonium chloride solution and dead cells were partially removed by centrifugation through foetal calf serum.

*B cells.* TNP-primed B cells were prepared from spleens of mice immunized with either TNP-OVA or TNP-FGG. T cells were removed by treatment with a rabbit anti-Thy 1 antiserum and guinea-pig complement. Erythrocytes and dead cells were removed by the methods described previously.

*Preparation of concanavalin A supernatants*

Normal CBA spleen cells were cultured for 24 hr with Con A (2 $\mu$g/ml). Culture fluid supernatants were then

harvested and the Con A concentration was reduced by passage of the supernatants through a Sephadex G50 column (Rich & Pierce, 1974). Supernatants were stored at −20° until use.

*General plan of the experiments and culture conditions*

The experimental conditions have been described elsewhere (Lefkovits & Waldmann, 1979). Essentially, $10^4$–$10^5$ KLH-primed spleen cells and $1–2 \times 10^5$ TNP-primed B cells were co-cultured with various antigens in 10 μl volumes in Terasaki microculture wells. Details of antigen concentrations and the source of T and B cells are given in the table legends. For each experimental group, 30 separate cultures were established and maintained at 37° in an atmosphere of 10% $CO_2$. The culture medium was Eagle's minimum essential medium supplemented with various additives including foetal calf serum (5%). After a 5 day culture period, IgM and IgG anti-TNP plaque-forming cells (PFC) in individual culture wells were assayed by a modification of the Jerne plaque technique, using TNP-conjugated donkey erythrocytes as the target antigen. The results are presented as the total number of IgM plus IgG anti-TNP PFC in 30 wells.

## RESULTS

Co-operation between T and B lymphocytes was analysed in the *in vitro* secondary antibody response to TNP-conjugated soluble protein antigens. Various sources of carrier-primed cells were used and antibody responses were measured when haptenic and carrier determinants were either linked or non-linked.

### Unpurified KLH-primed spleen cells can co-operate through both linked and non-linked recognition mechanisms

Initially, unpurified, mitomycin C-treated, spleen cells from mice immunized with KLH plus adjuvant were used as a source of T helper cells. These cells were co-cultured with TNP-primed B cells (anti-Thy 1-treated spleen cells), stimulated with various

**Table 1.** KLH-primed spleen cells co-operate with primed B cells when hapten and carrier determinants are both linked and non-linked

| TNP-primed* B cells | KLH-primed spleen† cells | Antigen‡ | Experiment no. 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|---|
| + | − | TNP-FGG+ 20 μg/ml KLH | 100§ | 250 | nt | nt | nt |
| | | TNP-OVA+ 20 μg/ml KLH | nt | nt | 190 | 180 | 136 |
| + | + | TNP-KLH | 7970 | 2875 | 1150 | 700 | 3947 |
| | | 20 μg/ml KLH | nt | nt | 400 | 140 | nt |
| | | TNP-OVA | 450 | 230 | 260 | nt | 183 |
| | | TNP-OVA+ 20 μg/ml KLH | 1980 | 400 | 870 | 400 | 1172 |
| | | TNP-FGG+ 20 μg/ml KLH | 3650 | 1400 | 660 | nt | nt |
| | | TNP-OVA+ 0·4 μg/ml KLH | nt | nt | nt | nt | 338 |

* TNP-primed B cells were anti-T cell treated spleen cells from CBA mice primed and boosted with either TNP-FGG (experiments 1 and 2) or TNP-OVA (experiments 3, 4 and 5). These were added at $1·5 \times 10^5$/well.

† KLH-primed spleen cells were treated with mitomycin C only. These were added at $1 \times 10^5$/well.

‡ The antigen concentrations in culture were as follows: TNP-KLH 0·4 μg/ml; TNP-OVA 1–2 μg/ml; TNP-FGG 2 μg/ml; KLH 20 μg/ml or 0·4 μg/ml.

§ IgM and IgG anti-TNP PFC were measured on day 5. Results are expressed as the total number of PFC in 30 culture wells.

antigens and the anti-TNP PFC response was measured (Table 1). Participation of both T and B cells was essential, since no response was seen in cultures lacking T cells or in cultures stimulated with either the carrier antigen only or with TNP coupled to an irrelevant carrier. Antibody responses were maximal when the relevant haptenic and carrier determinants were covalently associated (i.e., in cultures stimulated with TNP-KLH). However, substantial antibody responses were also elicited by challenge with antigenic determinants on separate molecules (i.e., TNP-OVA or TNP-FGG plus KLH). A high concentration of the carrier antigen (20 μg KLH/ml) was necessary to elicit non-linked help. This will be discussed in more detail later. Thus, using unpurified KLH-primed spleen cells as a source of help, both linked and non-linked interaction mechanisms can contribute to antibody responses. Similar results were obtained with B cells from animals boosted from 2 to 18 weeks before they were killed (data not shown). Thus capacity for non-linked recognition interactions is not restricted to recently boosted B cells.

It has previously been shown that the source of T cells influences the frequency of T helper cells with functionally distinct properties, such as the ability to mediate linked or non-linked recognition interactions (Waldmann & Pope, 1977; Marrack & Kappler, 1976). In the next series of experiments, the relative contributions of these two co-operative mechanisms were measured in cultures containing different T cell preparations.

**Nylon wool-non-adherent T cells co-operate mainly through linked recognition interactions**

T cells from mice immunized with KLH plus adjuvant and purified by nylon wool filtration have previously been shown to efficiently help B cells respond to soluble protein antigens. Such T cells were employed in this series of experiments. Cultures containing nylon wool nonadherent T cells and TNP-primed B cells were stimulated with various antigens and the anti-TNP response was measured (Table 2). In five experiments (Table 2, Exps 1–5) antibody responses were elicited only when the relevant antigenic determinants were covalently linked. Thus nylon wool-filtered T cells co-operated efficiently with B cells only through a linked recognition mechanism. In two experiments (Exps 6, 7) a substantial response was obtained on stimulation with unlinked hapten and carrier. However, in both these instances, the B cell population alone made a substantial response, suggesting that depletion of T cells was incomplete.

**Educated thymocytes co-operate through non-linked recognition interaction**

A different result was obtained when a third source of T helper cells was employed. Thymocytes were 'educated' by exposure to KLH in irradiated mice and the spleen cells of these mice were used as a source of T helper cells in similar experiments. (T cells prepared in this way have previously been shown to be good sources of non-antigen-specific T helper factors.)

**Table 2.** Nylon wool-filtered T cells co-operate with B cells only when haptenic and carrier determinants are linked

| TNP-OVA-primed B cells | KLH-primed T cells | Antigen | Experiment no. 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|---|
| + | – | TNP-OVA | 20* | 70 | 0 | 250 | 100 | 650 | 500 |
| + | + | TNP-KLH | 2950 | 1710 | 2230 | 2030 | 3300 | 3320 | 5410 |
| | | TNP-OVA | 310 | 310 | nt | 70 | nt | 430 | 950 |
| | | KLH | 390 | 270 | nt | nt | 320 | 1100 | 2300 |
| | | TNP-OVA+KLH | 310 | 250 | 90 | 350 | 420 | 2150 | 2700 |
| | | TNP-FGG+KLH | 230 | 170 | nt | nt | nt | nt | nt |

* Results are expressed as anti-TNP PFC in 30 wells.

T cells were nylon wool-passed, mitomycin C-treated KLH-primed spleen cells. Cell concentrations as in Table 1. Antigen concentrations were as noted in Table 1 (except KLH which was present at 20 μg/ml only). Results obtained at one T cell concentration only are presented but responses were assayed over a range of T cell concentrations and similar effects were observed.

**Table 3.** 'Educated' thymocytes co-operate equally efficiently when antigenic determinants are linked and non-linked

| B cells | KLH-primed T cells | Antigen(s) | Anti-TNP PFC/30 wells Exp. 1 | Exp. 2 |
|---|---|---|---|---|
| + | – | TNP-OVA | 140 | 140 |
| + | Educated thymocytes | TNP-OVA | 270 | 140 |
| | | 20 μg/ml KLH | nt | 430 |
| | | TNP-OVA + 0·4 μg/ml KLH | 130 | nt |
| | | TNP-OVA + 20 μg/ml KLH | 620 | 960 |
| | | TNP-KLH | 1200 | 1170 |
| + | Nylon wool-passed spleen cells | TNP-OVA | 180 | 400 |
| | | 20 μg/ml KLH | nt | 350 |
| | | TNP-OVA + 20 μg/ml KLH | nt | 460 |
| | | TNP-KLH | 4000 | 3100 |

Thymocytes, educated by exposure to KLH in irradiated hosts, were treated with mitomycin C before co-culture with TNP-primed B cells. Nylon wool-filtered, mitomycin C-treated spleen cells from KLH-primed mice were also co-cultured with B cells in these experiments. Cell concentrations were as Table 1. Antigen concentrations were as follows: TNP-OVA 1 μg/ml; TNP-KLH 0·4 μg/ml; KLH 0·4 μg/ml or 20 μg/ml.

Antibody responses were similar whether haptenic and carrier determinants were linked or non-linked (Table 3). Thus T cells from this source do not require linked recognition of antigenic determinants for co-operation. However, PFC responses in these cultures were relatively low. In these experiments, nylon wool-non-adherent T cells from KLH-primed mice were also co-cultured with B cells and various antigens. These T cells facilitated a considerably greater PFC response than did educated thymocytes, provided that haptenic and carrier determinants were linked. Thus linked recognition co-operation appears to be the most efficient helper mechanism in this system.

### High carrier concentrations are necessary for non-linked recognition co-operation

Non-linked recognition interactions have previously been studied extensively in *in-vitro* responses to particulate antigens. In general stimulation of T cells with high antigen concentrations is esential to elicit this helper function (Waldmann & Munro, 1974; Marrack and Kappler, 1976). In this study, stimulation of T cells with similarly high antigen concentrations was found to be necessary for non-linked recognition co-operation in the response to soluble protein antigens also. A low antigen concentration ($< 1$ μg/ml TNP-KLH) could only elicit an optimal antibody response when hapten and carrier were linked. Under these circumstances increasing the antigen concentration did not increase the response (data not shown). In contrast, high concentrations of the carrier antigen (20 μg/ml KLH) facilitated non-linked recognition interactions whereas low concentrations of carrier antigen (0·4–1 μg/ml KLH) failed to do so. This was observed in cultures containing either KLH-primed spleen cells (Table 1) or educated thymocytes (Table 3). A constant hapten (TNP-OVA) concentration was used in all experiments.

### Soluble factors replace the requirement for T cells

Many reports in the literature indicate that non-antigen-specific soluble factors mediate non-linked recognition interactions in responses to particulate antigens. We examined whether such factors are also

**Table 4.** Con A supernatant partially replaces the requirement for T cell help

| B cells | KLH-primed T cells | Con A supernatant | Antigen | Experiment no. 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|---|
| + | − | − | +TNP-OVA | 0* | 69 | 0 | 27 |
| + | − | + | +TNP-OVA | 1266 | 678 | 235 | 180 |
| + | − | + | +TNP-KLH | 1195 | 652 | 532 | 330 |
| + | + | − | +TNP-KLH | 1680 | 1714 | 1760 | 637 |

* Results are expressed as anti-TNP PFC in 30 wells.

Con A supernatant was present at a final concentration of 50%. TNP-OVA-primed B cells were added at $2 \times 10^5$/well. KLH-primed T cells, added at $1 \times 10^5$/well, were nylon wool-passed mitomycin C-treated spleen cells. Antigen concentrations were: TNP-OVA 1 μg/ml; TNP-KLH 0·4 μg/ml.

involved in the helper effects studied here. The capacity of supernatants of concanavalin A-activated spleen cells (Con A supernatant) to replace the requirement for T helper cells was assessed. T cell-depleted TNP-primed B cells were cultured with various antigens and Con A supernatant (Table 4). Responses facilitated by KLH-primed T cells were also measured in the same experiment. Con A supernatant induced a response approximately 30–60% of that observed with helper T cells, indicating that a proportion of primed B cells were capable of responding to non-antigen-specific helper factors. In cultures containing Con A supernatant there was no difference in the magnitude of the response stimulated by the homologous TNP-protein conjugate (i.e., that used to prime the B cells) and a heterologous TNP-conjugate. This suggests that Con A supernatant did not function primarily through expansion of contaminating antigen-specific T cells, although involvement of such residual T cells cannot be excluded.

## DISCUSSION

The data in this paper demonstrate that T and B cells responding to soluble protein antigens can interact through at least two mechanisms, one involving linked recognition of antigenic determinants and the second involving non-linked recognition. When responses are assayed using similar culture conditions and B cell preparations, the relative importance of each mechanism depends on the mode of priming and preparation of the T cells, and also on the concentration of the carrier antigen. A number of other studies have demonstrated that the stage of B cell differentiation influences the nature of the activation signals required (Andersson, Schreier & Melchers, 1980; Asano, Singer & Hodes, 1981). Thus many different factors affect the mechanisms by which T and B cells co-operate.

Linked recognition co-operation is the most efficient mechanism in the secondary antibody responses analysed here, since the highest responses were always elicited when haptenic and carrier determinants were linked. Non-linked recognition interactions seem to be of secondary importance but are clearly capable of contributing substantially to antibody responses under certain conditions. Thus generation of antibody responses is frequently a complex event, likely to be significantly influenced by (apparently) trivial differences in experimental systems. The non-linked recognition interactions investigated here are probably mediated by non-antigen specific soluble T-cell factors. Stimulation of educated thymocytes or KLH plus adjuvant-primed cells with high antigen concentrations has prevously been shown to result in release of such factors (Waldmann & Munro, 1974). Demonstration of partial replacement of T cell help by a Con A supernatant also supports this hypothesis.

In experiments reported here, unpurified primed spleen cells co-operated with B cells through both linked and non-linked recognition mechanisms. In contrast, nylon wool-filtered T cells co-operated primarily through linked recognition interactions. One interpretation of this result is that a subpopulation of T cells mediating non-linked recognition co-operation is nylon wool-adherent. Direct evidence for this has been reported by Tada *et al.* (1978). This hypothesis is further corroborated by indirect evidence from a number of other studies; some authors who reported

non-linked recognition co-operation in the response to soluble antigens have used unseparated spleen cell populations (Hunig *et al.*, 1977) or removed B cells by depletion with anti-Ig antibodies (Takatsu *et al.*, 1980). Thus both nylon wool-adherent and non-adherent T cells were present. In other studies where non-linked recognition interactions could not be demonstrated, nylon wool-purified cells were the source of T helper function (Henry, 1975). The probability that nylon wool filtration results in loss of a functionally significant helper T cell population is clearly important, in view of the very frequent use of this methodology.

Non-linked recognition interactions required substantially higher antigen concentrations than did linked recognition interactions. (It is noteworthy that generation of antibody responses in this microculture system requires substantially higher antigen concentrations than in more conventional Mishell-Dutton systems. Nonetheless the same general rule applies—linked recognition co-operation occurs at lower antigen concentrations than does non-linked recognition co-operation.) Hunig *et al.* (1977) and Takatsu *et al.* (1980) report similar findings. This difference in stimulation requirements is of practical value since it permits analysis of linked recognition interactions whilst minimizing non-linked recognition co-operation. However the dependence of non-linked recognition on high antigen concentrations may have physiological significance. Possibly non-specific soluble mediators function primarily as amplifiers. Thus T cells may release such factors only when stimulated by high antigen concentrations and only activated B cells may respond (as suggested by Andersson *et al.*, 1980). Possibly linked recognition interactions may deliver an essential first signal which renders B cells responsive to a second amplifying signal, such as TRF, delivered by non-linked recognition interactions. Indeed, evidence for synergy between these two mechanisms has been reported (Tada *et al.*, 1978; Keller *et al.*, 1980).

In conclusion, it is clear that generation of antibody responses is a complex event in which alternative or synergizing pathways may operate. Many distinct factors influence which mode of interaction predominates. This study provides information on the effects of T cell heterogeneity and antigen concentrations. Linked recognition iteractions are favoured when nylon wool-purified T cells and low antigen concentrations are employed. High antigen concentrations and the presence of nylon wool-adherent T cells favour non-linked recognition interactions. However, since the mode of interaction is influenced by many non-quantifiable factors, it is probably necessary to establish the basic mechanisms of T–B co-operation in each individual experimental system.

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