Immunology 1976 **30** 181

Estimation of hapten-specific antibody-forming cell precursors in microcultures*

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Received 13 *June* 1975; *accepted for publication* 29 *August* 1975

Summary. The immune response of mouse spleen cells to hapten-conjugated polymer of flagellin (DNP–POL, NIP–POL) was studied using a microculture system. When increasing numbers of spleen cells were added to a 'filler' cell system, negative feedback effects became apparent and resulted in the generation of progressively lower numbers of plaque-forming cells (PFC) per input cell. This feedback inhibition was shown to be antigen-specific and mediated by factors released into the culture medium. The effect precludes calculation of the frequency of PFC precursors in cultures containing spleen cells alone and complicates the analysis of tolerance using *in vitro* assay systems.

The addition of small numbers of spleen cells to a constant number of thymocytes provided a system in which Poisson analysis could be used to determine the frequency of PFC precursors capable of being activated by hapten–POL conjugates. This system was used to estimate the frequency of anti-NIP–PFC precursors in CBA spleen cells.

INTRODUCTION

A key assumption of cellular immunology has been that the magnitude of an immune response, measured by the number of antibody-forming cells (AFC) appearing after antigenic stimulation, relates directly to the number of competent specific precursor cells (AFCP) in the cell population entering assay. Manipulations are said to have caused tolerance when they result in the specific decrease in the AFC numbers obtained. This has been shown to be an unjustified assumption for an adoptive transfer assay even under conditions where the availability of T cells is not limiting (Stocker, Osmond and Nossal, 1974) and the use of this system to draw conclusions about the tolerant state is further complicated by recovery of responsiveness (Stocker and Nossal, 1974) in the adoptive hosts.

In vitro assays have the advantage of being a closed system where cells cannot enter and leave, and in which the AFC arise earlier than under adoptive transfer conditions.

Specific suppression of an immune response *in vitro* following the addition of antibody or immune complexes is well known. This report describes specific feedback suppression arising within microcultures which contain spleen cells at a cell and antigen concentration chosen to give peak AFC responses. The conclusion is reached that these effects are of sufficient magnitude to compromise seriously, interpretation of many tissue culture experiments. A modification of the system which allows single-hit kinetics is described and an estimate of the number of spleen B cells reactive to a hapten is made.

* This is publication number 2114 from The Walter and Eliza Hall Institute of Medical Research.

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MATERIALS AND METHODS

Mice

Male CBA Ca/H. Wehi mice aged 7–9 weeks, homozygous nude mice nu/nu and heterozygous nu/+ littermates on a BALB/c An. Bradley Wehi background were obtained from a specific pathogen-free colony. The nude mice had undergone five to seven back-cross generations to the BALB/c strain. Male (C57Bl/6J × CBA/H. Wehi)F1 hybrids aged 8 weeks were used in some experiments.

Tissue culture

Spleen cells were cultured in microplates (flat-bottomed Microtest II tissue culture plate, System Cooke, Greiner, Germany) using a modification of the system of Lefkovits (1972). The culture conditions are described in detail elsewhere (Pike, 1975). Briefly, cells were cultured in HEPES-buffered Eagle's minimal essential medium (HEM) containing 10^{-4} M 2-mercaptoethanol, 5 per cent foetal calf serum (FCS) and the test antigen with 10^6 viable nucleated cells added to each well in a volume of 0·2 ml, except when thymus 'filler' cells were used where 2×10^6 cells/0·2 ml were usually added. The cultures were incubated at 37° in a humidified atmosphere containing 10 per cent CO_2 in air for 48–72 h. The contents of each well were transferred to a centrifuge tube using a Pasteur pipette and the volume of cell suspension was made up to 5 ml with HEM. The cells were recovered for the plaque assay by centrifugation.

In some experiments cells were cultured in trays (20-mm square compartment tissue culture trays) (Filtrona Plastics, Melbourne). Spleen cells were suspended in medium at 1×10^7/ml and 2·0-ml suspension was added to each well. Otherwise the culture conditions were identical with those of the microculture system.

Antigens

Dinitrophenylated polymerized flagellin (DNP–POL) was prepared by the method of Feldmann (1971), from POL supplied by Mr J. Pye. NIP–POL was prepared by coupling POL with NIP azide using the method of Brownstone, Mitchison and Pitt-Rivers (1966). The substitution ratios of hapten per mole of monomeric flagellin in POL were 1·4:1 and 1·6:1 for DNP and NIP:POL respectively. DNP–HGG was prepared by the method of Eisen (1964).

Plaque assay

This was performed by the method of Cunningham and Szenberg (1968) using sheep erythrocytes (SRBC) coated with DNP-conjugated Fab fragments of rabbit anti-SRBC to detect anti-DNP antibody-forming cells (AFC).

For the detection of anti-NIP–AFC, NIP-conjugated Fab fragments were prepared by the method of Brownstone *et al.* (1966).

Plaque inhibition test

The H-2 type of AFC was tested in some experiments by incubation of the cell suspension with anti-H 2 antisera and complement, prior to assay for PFC. The method for preparation of antisera and conditions for cell incubation were those of Mitchell and Miller (1968).

Tolerance induction

Mice were rendered tolerant to DNP by a single intravenous injection of 5·0 mg of DNP_1–HGG (Stocker and Nossal, 1974). Aggregates were removed from the preparation immediately prior to injection by centrifugation at 100,000 *g* for 90 min (Chiller, Habicht and Weigle, 1971). Spleen cells were used from these mice between 7 and 14 days after injection. Mice from each group were tested by assaying the spleens for anti-DNP–PFC 4 days after receiving 5 μg of DNP–POL by intraperitoneal (i.p.) injection. The number of anti-DNP–PFC from these mice was always less than 5 per cent of the response of saline pre-treated controls, which yielded $4–6 \times 10^4$ PFC per spleen.

Statistics

Probability values were calculated using Student's *t*-test. For limit dilution experiments in which PFC arose from both the spleen cell population and from the thymocyte filler cells, the probability of a culture containing no precursor cell was assumed to follow a Poisson distribution (Quintans and Lefkovits, 1973).

With the following definitions: μ_t = precursor frequency in constant number of 2×10^6 thymus cells; μ_s = precursor frequency in unit number of added spleen cells; n = number of added spleen cells; the probability of a culture containing no PFC (P) is given by the relationship $P = e^{-(n\mu_s + \mu_t)}$ This leads to

$$\log_e \frac{1}{P} = \mu_t + n\mu_s.$$

Thus, if the assumptions are correct, $\log_e 1/P$ should be linearly related to n, with the gradient of the line providing the value μ_s.

RESULTS

Challenge of normal and tolerant CBA spleen cells with DNP–POL or NIP–POL *in vitro*

1×10^6 spleen cells from normal or tolerant CBA mice were cultured with DNP–POL or NIP–POL or both antigens (Fig. 1). The lack of cross-reactivity

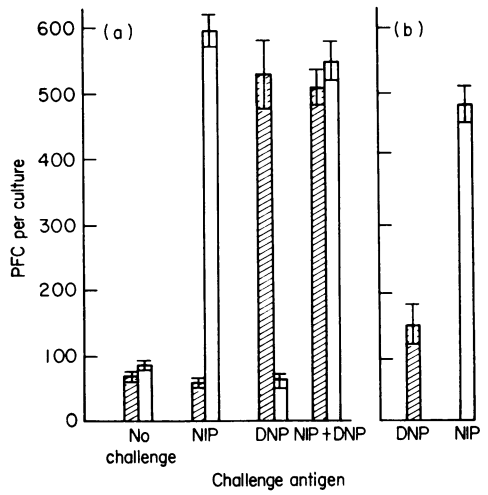

Figure 1. Effect of microculture challenge with DNP–POL or NIP–POL on spleen cells from (a) normal mice or (b) mice tolerant to DNP. Hatched columns, anti-DNP–PFC, open columns, anti-NIP–PFC.

between the antigens is apparent, with no elevation above the PFC number obtained from background unstimulated cultures occurring in cultures stimulated with NIP–POL and assayed for anti-DNP–PFC and *vice versa*. No antigenic competition between the two haptens was noted. Spleen cells from tolerant mice consistently showed a 3–6-fold reduction in responsiveness to DNP–POL. This level of responsiveness is considerably higher than that obtained from *in vivo* challenge of tolerant mice with DNP–POL. Such animals always show less than 5 per cent of the PFC response of controls (Stocker and Nossal, 1974).

Effect of adding normal spleen cells to 1×10^6 tolerant spleen cells

In an attempt to measure the response of a small number of spleen cells in a 'filler' cell system functionally depleted of precursors, spleen cells from normal mice were added in various numbers to cultures in which a constant number (1×10^6) of tolerant spleen cells was present. The cultures were stimulated with DNP–POL. Table 1 shows the results of two typical experiments.

Table 1. Addition of normal spleen cells to tolerant spleen cells in microcultures challenged with DNP–POL

Number of normal spleen cells added per culture ($\times 10^{-3}$)	PFC per culture ± s.e.m.	
	Expt 1	Expt 2
0	39±8	79±12
10	64±14	83±17
20	72±16	157±32
40	94±14	159±29
80	140±25	146±17
160	165±15	272±30
320	267±14	346±32
1000	475±40	475±46

The number of PFC obtained from these cultures was increased by the addition of normal spleen cells and was clearly above the background level of tolerant cells with the addition of 40×10^3 normal cells ($P < 0.005$ for experiments 1 and 2). Using this system, a plateau level was usually reached with the addition of 1×10^5–3×10^5 normal spleen cells, with a proportionately smaller rise in PFC above this number. To show that this was not simply due to cell crowding, 1×10^6 normal spleen cells were cultured with 1×10^6 tolerant cells. This culture yielded 490±42 PFC. Thus it appeared that a suboptimal response was obtained from normal cells at higher cell dose. This suggested that negative feedback mechanisms may have been limiting the response of 1×10^6 normal spleen cells.

Source of responding cells

The source of additional PFC in cultures of tolerant cells to which normal spleen cells were added,

was determined using the anti-H 2 and complement plaque inhibition assay. (C57Bl/6J × CBA)F1 hybrid mice were used as the source of tolerant spleen cells and 1×10^5 CBA nude spleen cells were added. The cells recovered were incubated with CBA anti-C57Bl/6J serum and complement prior to assay. Fig. 2 shows that the additional PFC arising in such cultures are derived from the added CBA nude cells.

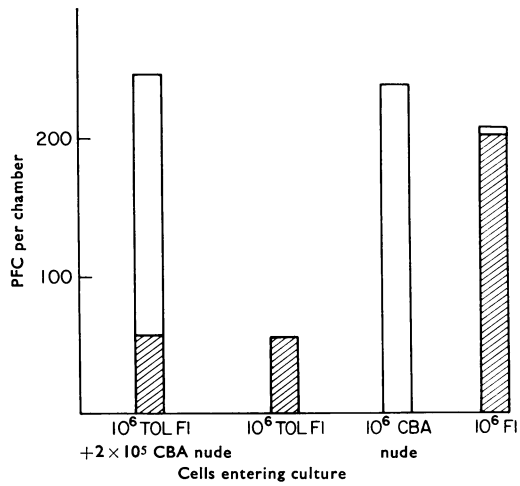

Figure 2. Effect of CBA anti-C57Bl antiserum plus complement on the PFC arising in microcultures containing tolerant (CBA × C57Bl)F1 hybrid spleen cells to which CBA nude spleen cells were added. Hatched portion of columns, PFC inhibited by antiserum plus complement.

In similar experiments where normal CBA spleen cells were added to the F1 hybrid cells, additional PFC were derived from both tolerant and normal populations (unpublished observations). This suggested that some of the tolerant spleen cells may be 'rescued' in the presence of an allogenic effect.

The possibility that antigen-specific feedback was limiting the response of larger cell numbers due to factors released into the medium was then investigated.

Effect of supernatant medium from spleen cell cultures responding to DNP–POL

CBA spleen cells from normal or from tolerant mice were cultured in trays (see Materials and Methods section) at a cell density of 2×10^7 cells in 2 ml of medium. The cultures were stimulated with 100 ng/ml of DNP–POL. After 3 days the cells were harvested by centrifugation and assayed for anti-DNP–PFC.

The PFC numbers were 776 ± 36 (mean ± s.e.m.) and 56 ± 5, for normal and tolerant cells respectively.

The supernatant medium from these cultures was tested for its capacity to support the response of fresh CBA spleen cells to DNP–POL and to NIP–POL in microcultures. The medium was used in dilutions and groups of cultures were assayed at 48 and 72 h.

Fig. 3 shows the effect of undiluted supernatant. Supernatant from normal spleen cells responding

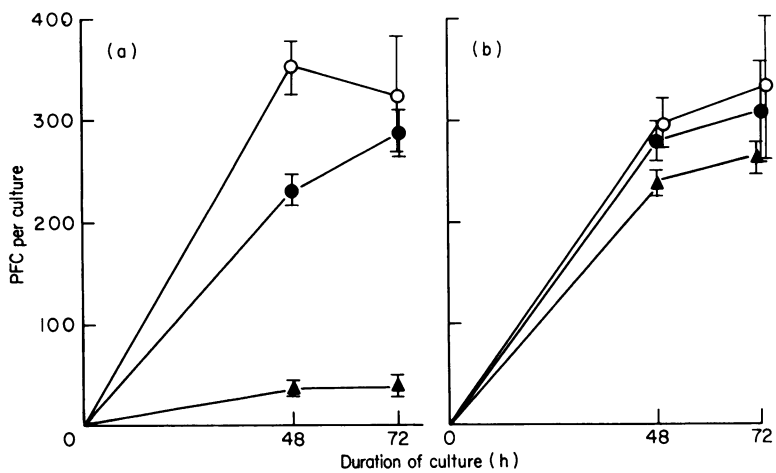

Figure 3. Effect of supernatant media on the response to DNP. (○) Normal culture medium; (●) supernatant medium from cultures of spleen cells from mice tolerant to DNP; (▲) supernatant medium from normal spleen cells. (a) DNP–PFC. (b) NIP–PFC.

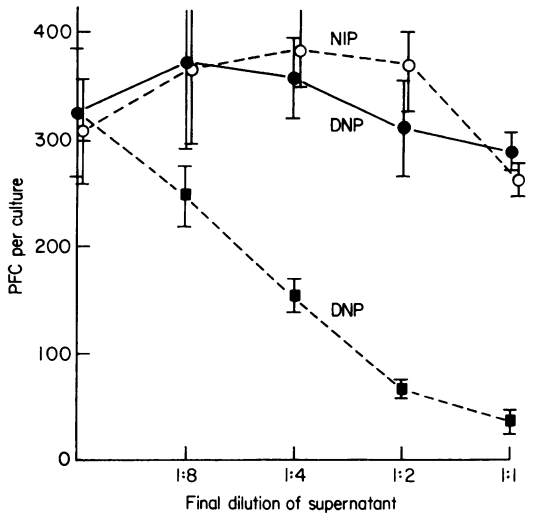

Figure 4. Effect of various dilutions of supernatants on the microculture response to DNP. (– – –) Anti-NIP (○) and anti-DNP (■) response in cultures containing supernatant from normal spleen cells. (———) Anti-DNP-PFC (●) from cultures containing supernatant medium from tolerant spleen cells.

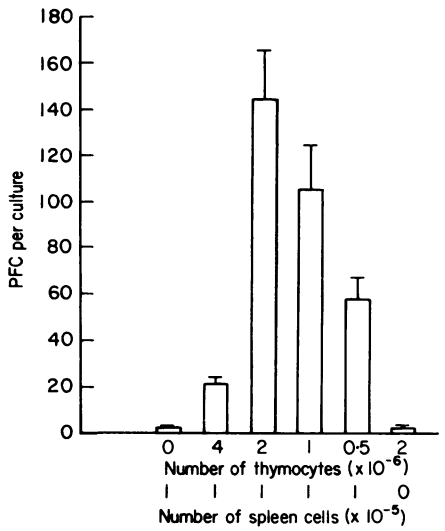

Figure 5. Effect of culturing spleen cells in the presence of various numbers of syngeneic thymocytes.

to DNP–POL was highly suppressive to the anti-DNP response in the microculture and medium taken from tolerant cells was slightly suppressive when the assay was performed at 48 h. The anti-NIP response was not significantly depressed by either medium.

Fig. 4 shows the effect of various dilutions of these supernatant media at 48 h. The DNP-specific suppressive effect of medium from normal spleen cells is progressively diluted out. Slight depression of the anti-NIP response observed with undiluted supernatant is removed by 1:2 dilution.

Effect of culturing small numbers of CBA spleen cells in the presence of thymocytes

The background response from the spleen cells of tolerant mice rendered this a suboptimal system to provide 'filler cells' for the assay of PFC precursors in a small number of added spleen cells. Irradiated cells and cells treated with mitomycin are also ineffective filler cells in this culture system (Haas and Layton, 1975). Cells from the thymus of normal CBA mice were used as a source of cells capable of division but in which few B cells would be expected.

Fig. 5 shows the results of adding various numbers of thymocytes to 1×10^5 normal spleen cells in

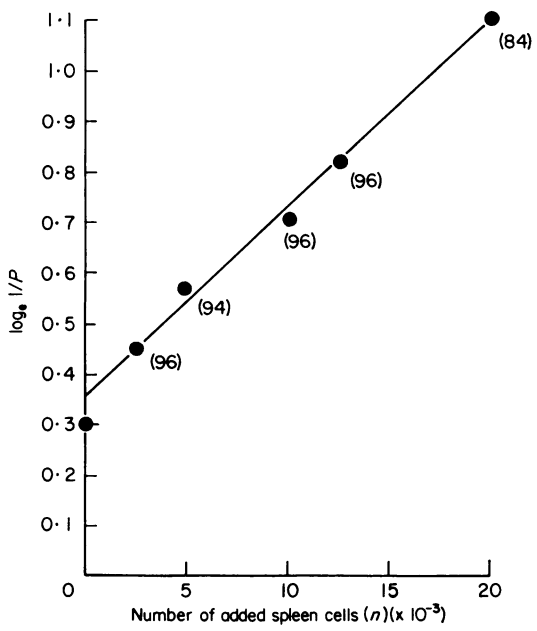

Figure 6. Plot of $\log_e 1/P$ versus spleen cell number added to cultures containing $2{\cdot}0 \times 10^6$ thymocytes. Figures in parentheses represent the number of cultures assayed at each point.

microcultures challenged with NIP–POL. Thymocytes support a response in this system which is optimal for 2×10^6 thymocytes added per well. There

is a very low background response from the thymocytes. In this and other experiments where $0–1·0 \times 10^5$ spleen cells were added to $2·0 \times 10^6$ thymus cells and challenged with NIP–POL, no anti-SRC–PFC were detected. Hence in subsequent experiments, only NIP–SRC were used in the plaque revelation system.

Use of thymocyte filler cell system to estimate the frequency of precursors of anti-NIP–PFC

Small numbers ($0–2·0 \times 10^4$) of CBA spleen cells were added to cultures containing $2·0 \times 10^6$ CBA thymocytes. The cultures were challenged with NIP–POL and assayed at 72 h for anti-NIP–PFC. The frequency of cultures in which no PFC were detected was calculated (P) and $\log_e 1/P$ was plotted against the number of spleen cells added to the culture (see Materials and Methods section). Fig. 6 shows that a linear relationship exists between these parameters. The gradient of the line is $3·6 \times 10^{-5}$. This experiment was repeated twice with gradients of $3·0 \times 10^{-5}$ and $7·95 \times 10^{-5}$ being obtained.

DISCUSSION

The microculture system provides a convenient means of generating an immune response *in vitro* (Lefkovits, 1972; Pike, 1975), and is a highly sensitive system with an efficiency greater than that of Marbrook flask cultures (Pike, 1975). The present study shows that when spleen cells are cultured in a concentration of cells and a T cell-independent antigen at a dose chosen to give peak response, the number of PFC obtained is lower than that expected from the number of precursors in the population. By adding small numbers of spleen cells to a 'filler' cell population, itself low in B-cell content, the immune response can be studied in a cell dose range where the number of PFC generated is linearly related to the precursor cell frequency, free of suppressive effects.

The regulatory effect of antibody on the immune response *in vivo* is well known (reviewed by Uhr and Möller, 1968). Similarly the immune response *in vitro* can be suppressed by the addition of immune complexes (Diener and Feldmann, 1970; Dennert, 1973) or specific antibody (Pierce, 1969; Radcliffe and Axelrad, 1971; Schrader, 1973). The present demonstration that specific suppression limits the number of PFC arising in cultures of spleen cells, complicates the use of the system to assay for the precursors of specific PFC. A difference in the numbers of PFC derived from cultures of normal versus tolerant cells may provide a reflection of these control mechanisms rather than providing information about the frequency of competent precursors. Similarly, antibody-mediated feedback may be the basis for some of the reported systems in which T cell-dependent suppression of an *in vitro* immune response occurs (e.g. Huchet and Feldmann, 1974) and suppression by 'excess help' may reflect the early production of specific antibody capable of mediating feedback effects (Basten, 1974).

For a T cell-dependent microculture system, Quintans and Lefkovits (1973) have shown that an estimate of precursor frequency can be obtained by limiting dilution analysis *in vitro* using spleen cells cultured in the presence of irradiated spleen cells and an allogenic cell stimulus. The function of various 'filler cell' populations in supporting an *in vitro* immune response is not understood. Thymus cells provide a population capable of supporting the immune response of a small number of added cells (Marbrook and Haskill, 1974), but possessing minimal 'background' problems.

The present experiments show that an estimate of the frequency of precursors of anti-NIP–PFC can be obtained by the addition of small numbers of spleen cells to thymocyte filler cells. The value of $3·0 \times 10^{-5}–7·95 \times 10^{-5}$ for the frequency of precursors of anti-NIP–PFC may be compared with the value of $1·1 \times 10^{-5}–5 \times 10^{-5}$ obtained for anti-SRBC–PFC precursors by Quintans and Lefkovits (1973).

Irradiated or mitomycin-treated cells do not provide optimal support conditions in the microculture system (Haas, 1975). Spleen cells in which a functional depletion of specific PFC precursors has been achieved using an immunoadsorbent (Haas, 1975) or spleen cells from tolerant mice, provide systems with a high background of PFC derived from the filler population. This background complicated the detection of the response of a small number of precursors in a population to be assayed. The thymus also contains small numbers of functional B cells, and it remains a major challenge to develop conditions under which B-cell activation and clonal expansion can be studied in the absence of filler cells and negative feedback control.

ACKNOWLEDGMENTS

The author is indebted to Dr J. D. Mathews for advice regarding statistics and to Miss Lucy Strzelecki for expert technical assistance. This work was supported by the National Health and Medical Research Council and the Australian Research Grants Committee, Canberra, Australia; by the Volkswagen Foundation (grant number 112147); and by the U.S. Public Health Service (grant number A 1-0-3958) and contract number NO1-CB-23889 with the National Cancer Institute, National Institutes of Health, Department of Health, Education and Welfare, U.S.A.

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