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Appearance of non-specific suppressor T cells during *in vitro* culture

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Summary. It was found that when normal mouse spleen cells were cultured for 4 days they were capable of non-specifically suppressing the *in vitro* antibody response of non-precultured spleen. The suppression was mediated by a subpopulation of viable, non-adherent, T lymphocytes with the surface phenotype Ly-1$^+$, Ly-2$^-$ and Ia$^-$. Furthermore, it appeared that the suppressor cells were responding to foetal calf serum antigens present in the tissue culture medium and were subsequently inactivating either B lymphocytes or accessory cells required for antibody formation, which had passively absorbed these antigens.

INTRODUCTION

In recent years numerous immunologists have used tissue culture techniques to examine immune responses. *In vitro* technology has also been used in this laboratory to examine several aspects of immune responsiveness. Whilst examining the *in vitro* antibody response to one antigen, DNP-MON, it became clear that after normal spleen cells had been cultured for 4 days in the absence of antigen they became unresponsive to DNP-MON. In fact, when these precultured cells were mixed with freshly prepared splenocytes they non-specifically suppressed the humoral response of the non-precultured cells.

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In this paper the nature of this non-specific immune suppression was examined more closely. It was found that the suppression was mediated by a T lymphocyte with the surface phenotype Ly-1$^+$, Ly-2$^-$ and Ia$^-$. Furthermore, it appeared that the suppressor cells were responding to foetal calf serum (FCS) antigens present in the tissue culture medium and were subsequently inactivating either B lymphocytes or accessory cells required for antibody formation, which had passively absorbed these antigens.

MATERIALS AND METHODS

Animals

Inbred CBA/H mice of either sex and from 6 to 14 weeks of age were used in these studies.

Antigens

Monomeric flagellin (MON) was prepared from the flagella of *Salmonella typhimurium* (SL870) organisms (Ada, Nossal, Pye & Abbot, 1964) and was dinitrophenylated as described by Eisen (1964) to give $DNP_{1\cdot5}MON$. Hemocyanin (HCY) was crystallized from the haemolymph of the South Australian crayfish, *Jasus lalandii*, and was also dinitrophenylated to form $DNP_{48}HCY$. Horse red blood cells (HRBC) were purchased from Commonwealth Serum Laboratories, Melbourne, and sheep red blood cells (SRBC) were harvested every second week from one sheep at the John Curtin School of Medical Research.

Immunizations

Mice were primed intravenously either 1–3 months before use with 10 μg of DNP-MON in 0·5 ml saline or 4 days before use with 2×10^5 HRBC in saline.

Antisera

The preparation and characteristics of the anti-Thy-1·2, anti-Ly-1·1 and anti-Ly-2·1 sera used in these studies have been reported previously (McKenzie & Parish, 1976; Vadas, Miller, McKenzie, Chism, Shen, Boyse, Gamble & Whitelaw, 1976). The anti-Ia serum was an A.TH anti-A.TL serum (anti-I^k) whose preparation and properties have already been described (McKenzie & Parish, 1976; Vadas *et al.*, 1976). We are indebted to Dr I. F. C. McKenzie for supplying the anti-Ly and anti-Ia reagents.

Preparation and fractionation of cell suspensions

Single cell suspensions of spleen cells were prepared as previously described (Parish, Kirov, Bowern & Blanden, 1974). Dead cells were removed from spleen cell preparations by centrifuging the cells on a cushion of Isopaque/Ficoll and harvesting the cells remaining at the Isopaque/Ficoll-medium interface (Davidson & Parish, 1975). In a similar manner spleen cells were separated into Ig^+ and Ig^- fractions (Parish *et al.*, 1974). Briefly, Ig^+ rosettes were formed by an indirect procedure and the rosetted cells separated from the non-rosetted cells by centrifugation on Isopaque/Ficoll. The non-rosetting (Ig^-) cells floated at the Isopaque/Ficoll-medium interface, whereas the rosettes (Ig^+ cells) sedimented to the bottom of the tube. Adherent cells were removed from cell suspensions by carbonyl iron treatment (Parish & Hayward, 1974). A two stage complement lysis procedure was used for eliminating spleen cell subpopulations with antisera (McKenzie & Parish, 1976). Guinea pig complement was used for the anti-Thy-1·2 treatments and rabbit complement for the anti-Ly and anti-Ia sera.

Tissue culture conditions

In most cases cells were prepared and cultured in Eagle's minimal essential medium (F-15; Grand Island Biological Co., Grand Island, N.Y.), buffered with sodium bicarbonate, and supplemented with 10% foetal calf serum (FCS), 100 μg/ml streptomycin, 100 units/ml penicillin G, and 10^{-4} M mercaptoethanol. The FCS was supplied by Commonwealth Serum Laboratories, Melbourne, and only one batch (no. 975.1) was used in the studies described. In a few cases 0·5% normal mouse serum (NMS—freshly prepared from adult mice) was used instead of FCS and in these instances the medium was also supplemented with the nucleic acid precursors guanosine, adenosine, cytosine and uridine at a final concentration of 25 μg/ml (Click, Benck & Alter, 1972). Cells were cultured with or without antigen (2·5 ml/well) in 16 mm Linbro trays (Linbro Chemical Co., New Haven, Conn., U.S.A.). In generating non-specific suppressor cells normal spleen cells were cultured for 4 days, in the absence of antigen, at a concentration of 2×10^6 cells/ml (5×10^6/well). The antigen concentrations used in the cultures were: DNP-MON, 100 ng/ml; DNP-HCY, 2 μg/ml; SRBC, 5×10^6/ml. Cultures were placed in a humidified incubator at 37° in an atmosphere of 10% CO_2, 7% O_2 in N_2 for varying times and then harvested and treated as required.

Enumeration of plaque-forming cells

Plaque-forming cells (PFC) were assayed by the technique of Cunningham & Szenberg (1968). To detect anti-DNP PFC, SRBC were coated with a dinitrophenylated rabbit anti-SRBC Fab, as described by Strausbauch, Sulica & Givol (1970). Only direct PFC were assayed.

RESULTS

Detection of non-specific suppressor cells in *in vitro* cultures

Whilst analysing the *in vitro* anti-DNP antibody response to DNP-MON it became apparent that normal spleen cells were unable to respond to DNP-MON if they were cultured for 4 days prior to addition of antigen. Furthermore, it was found that if these precultured cells were mixed with freshly prepared spleen cells they very effectively suppressed the anti-DNP response of the fresh splenocytes.

Figure 1 clearly demonstrates the immunosuppressive activity of the precultured cells. In this experiment graded numbers of precultured cells (suppressor cells) were mixed with a constant number (4×10^6) of freshly prepared normal spleen cells, DNP-MON added, and the subsequent anti-DNP PFC response measured. It can be seen that as few as $1{\cdot}25 \times 10^5$ precultured cells produced signi-

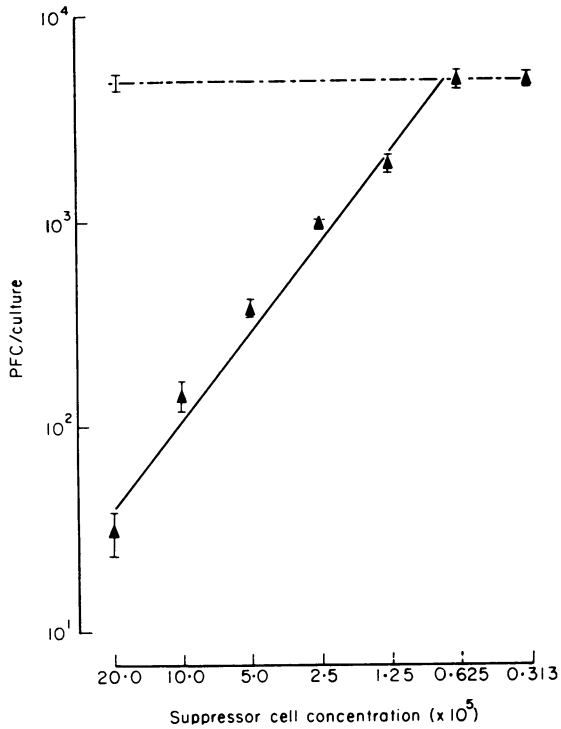

Figure 1. Ability of different numbers of normal spleen cells, which had been precultured for 4 days, to suppress the *in vitro* anti-DNP antibody response of 4×10^6 non-precultured spleen cells. PFC were assayed after 3 days culture and the stimulating antigen was DNP-MON. The discontinuous line represents the PFC response of 4×10^6 non-precultured spleen cells alone. In this experiment 6×10^6 non-precultured spleen cells gave 6225 ± 294 anti-DNP PFC/culture. Vertical bars represent standard errors of means.

ficant suppression and the extent of suppression steadily increased as more precultured cells were added, 2×10^6 suppressors producing a 99% reduction in the PFC response. These suppressive effects were not due to culture crowding as the addition of 2×10^6 freshly prepared spleen cells (rather than 2×10^6 precultured cells) actually augmented the anti-DNP response by almost 60%. Furthermore, the suppressor cells were not merely toxic as both the suppressed and non-suppressed cultures contained comparable numbers of viable cells.

It should be noted that a plot of the log of the antibody response versus the log of the number of suppressor cells added resulted in a straight line with a slope of 1·25 (Fig. 1). However, based on regression analysis, this slope was not significantly different from a slope of 1·0. This result suggests that a single population of suppressor cells is acting in this system.

The *in vitro* generated suppressor cells lacked antigen-specificity as they were capable of suppressing *in vitro* antibody responses to DNP-MON, DNP-HCY and SRBC (Table 1). In subsequent experiments it was found that secondary antibody responses to these three antigens were also strongly suppressed by the precultured cells. Also, the presence of immunogenic doses of DNP-MON during the preculture period in no way inhibited the generation of these non-specific suppressor cells (data not shown).

The rate of appearance of the suppressor cells

Table 1. Lack of antigen-specificity of *in vitro* generated suppressor cell

Spleen cell population		Antigen			PFC/culture‡	
Normal*	Suppressor†	DNP-MON	DNP-HCY	SRBC	Anti-DNP	Anti-SRBC
4×10^6	—	+	−	−	1720 ± 120	—
—	2×10^6	+	−	−	< 5	—
4×10^6	2×10^6	+	−	−	13 ± 5	—
4×10^6	—	−	+	−	528 ± 32	—
—	2×10^6	−	+	−	< 5	—
4×10^6	2×10^6	−	+	−	33 ± 9	—
4×10^6	—	−	−	+	—	628 ± 35
—	2×10^6	−	−	+	—	< 5
4×10^6	2×10^6	−	−	+	—	43 ± 16

* Represent freshly prepared spleen cell suspensions. With the DNP-MON response unprimed cells were used, whereas with the DNP-HCY and SRBC responses cells were cultured from either DNP-MON primed or HRBC primed mice.

† Spleen cells from normal (unprimed) mice which were cultured for 4 days before use.

‡ PFC responses after 4 days culture ± s.e.m.

Table 2. Demonstration of T cell nature of *in vitro* generated suppressor cell

Spleen cell population		Fraction of suppressor cells	Anti-DNP PFC/culture‡
Normal*	Suppressor†		
4×10^6	—	—	3878 ± 166
—	2×10^6	Unfractionated	< 5
4×10^6	2×10^6	Unfractionated	35 ± 7
4×10^6	$1{\cdot}8 \times 10^6$	Dead cells removed	20 ± 6
4×10^6	$1{\cdot}7 \times 10^6$	Adherent cells removed	55 ± 9
4×10^6	$0{\cdot}7 \times 10^6$	Ig^- cells	40 ± 9
4×10^6	1×10^6	Ig^+ cells	2813 ± 175
4×10^6	$1{\cdot}6 \times 10^6$	Complement treated	75 ± 9
4×10^6	$1{\cdot}2 \times 10^6$	Anti-Thy-1·2 + complement treated	1060 ± 24

* Cells freshly prepared from unprimed mice.

† Spleen cells from unprimed mice which were cultured for 4 days before use. Values represent viable cell numbers after treatment of 2×10^6 cells.

‡ PFC response after 3 days in culture with DNP-MON ± s.e.m.

Table 3. Characterization of T cell population which is immunosuppressive

Spleen cell population*		Treatment of suppressor cells	Anti-DNP PFC/culture†
Normal	Suppressor		
4×10^6	—	—	2070 ± 78
—	2×10^6	Untreated	< 5
4×10^6	2×10^6	Untreated	15 ± 6
4×10^6	$1{\cdot}5 \times 10^6$	Complement alone	63 ± 20
4×10^6	$0{\cdot}6 \times 10^6$	Anti-Ly-1·1 + complement	1473 ± 234
4×10^6	$1{\cdot}3 \times 10^6$	Anti-Ly-2·1 + complement	100 ± 24
4×10^6	$1{\cdot}2 \times 10^6$	Anti-Ia + complement	28 ± 11

* Normal and suppressor cells as in Table 2.

† PFC response after 3 days in culture with DNP-MON ± s.e.m.

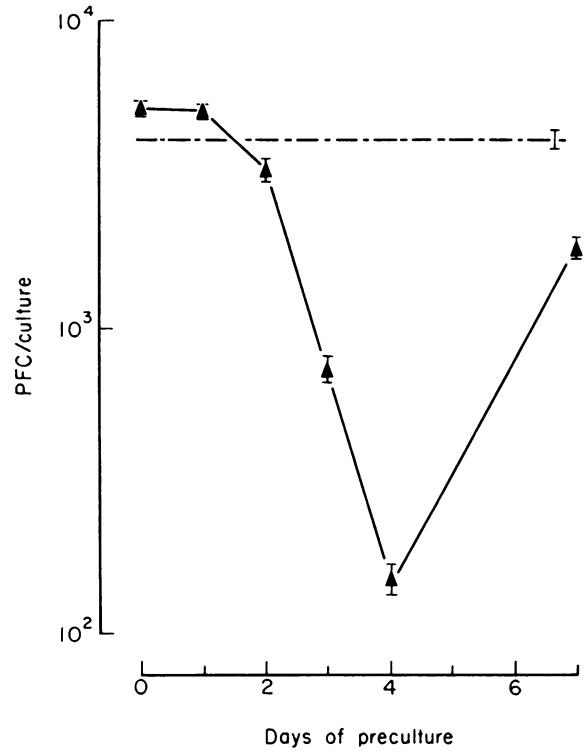

Figure 2. Time course of appearance of non-specific suppressor cells in *in vitro* culture. To 4×10^6 non-precultured spleen cells was added 2×10^6 spleen cells which had been precultured for varying times before addition. PFC were assayed after 3 days culture and the stimulating antigen was DNP-MON. The discontinuous line represents the PFC response of 4×10^6 non-precultured spleen cells alone. Vertical bars represent standard errors of means.

was then determined by preculturing normal spleen for varying times prior to mixing with non-precultured cells (Fig. 2). Substantial immunosuppression was detected after 3 days of preculture. Even higher levels of suppressor activity appeared after 4 days of culture but by 7 days of preculture this activity had, to a large extent, declined.

Characterization of non-specific suppressor cell

In the next series of experiments we attempted to identify the cell type responsible for the non-specific suppression. It was found that the suppressor was a viable, non-adherent cell whose suppressive activity was quantitatively recovered in the Ig^- population of spleen cells, i.e. it lacks surface immunoglobulin (Table 2). In contrast, anti-Thy-1·2 and complement treatment destroyed the bulk (approximately 90%) of the suppressor cells (Table 2). Furthermore, the suppressors were eliminated by anti-Ly-1·1 and complement treatment but were not destroyed by anti-Ly-2·1 and anti-Ia sera (Table 3). Collectively, these data indicate that the non-specific suppressor cell is a T lymphocyte with the surface phenotype $Ly\text{-}1^+$, $Ly\text{-}2^-$ and Ia^-.

It should be emphasized at this point that the same anti-Ia and anti-Ly sera used in these studies have been used previously to characterize different functional subsets of T lymphocytes (Vadas *et al.*, 1976; Beverley, Woody, Dunkley, Feldmann & McKenzie, 1976).

Mode of action of non-specific suppressor cell

The non-specific suppressor T cells which appear in *in vitro* cultures may well be activated by and directed against the FCS present in the tissue culture medium. In order to test this possibility normal spleen cells were precultured in tissue culture medium containing either FCS or NMS and the suppressive activity of these cells then assessed in secondary cultures which also contained either FCS or NMS.

Initially it was found that NMS (0·5%) could support a moderate anti-DNP PFC response to DNP-MON, i.e. approximately 25% of FCS (Table 4). However, when spleen cells were precultured in NMS they exhibited no suppressive activity and, in fact, tended to augment the anti-DNP response (Table 4). Cells precultured in FCS were, on the other hand, highly immunosuppressive

Table 4. Appearance and action of suppressor T cells is dependent upon foetal calf serum (FCS)*

Spleen cell population		Serum in culture medium of		
Normal	Suppressor	Suppressor culture	Anti-DNP culture	Anti-DNP PFC/culture†
4×10^6	—	—	FCS	2305 ± 133
4×10^6	—	—	NMS	590 ± 64
4×10^6	2×10^6	FCS	FCS	10 ± 4
4×10^6	2×10^6	NMS	FCS	3200 ± 94
4×10^6	2×10^6	NMS	NMS	708 ± 32
4×10^6	2×10^6	FCS	NMS	500 ± 41

* Suppressors generated by culturing unprimed spleen cells for 4 days in culture medium containing either 10 % FCS or 0·5 % CBA/H normal mouse serum (NMS). Subsequent anti-DNP cultures were in either 10 % FCS or 0·5 % NMS, as indicated.

† PFC response after 3 days in culture with DNP-MON ± s.e.m.

but only if the secondary culture medium also contained FCS (not NMS) (Table 4). From these results it was concluded that FCS is needed for the generation of the non-specific suppressor T cell and is also required during the effector phase of the suppression.

In additional experiments (data not shown) it was found that:

(a) Cells precultured in allogeneic NMS were not immunosuppressive.

(b) Cells precultured in medium containing as little as 0·3% FCS were still highly immunosuppressive. In addition, the component(s) in FCS which stimulated the generation of suppressor T cells were non-dialysable.

(c) The addition of up to 2×10^6 normal peritoneal cells/culture did not override the suppressive activity of the precultured cells.

DISCUSSION

Evidence is presented in this paper which indicates that when normal spleen cells are cultured for 4 days a suppressor cell appears which can non-specifically inhibit the antibody response of non-precultured spleen cells. The suppressor cell appears to be a viable, non-adherent, T lymphocyte with the surface phenotype $Ly\text{-}1^+$, $Ly\text{-}2^-$ and Ia^-. Thus, the non-specific suppressor cell belongs to a different

subset of T lymphocytes from the conventional, antigen specific, suppressors which have been demonstrated to be Ly-1$^-$, 2$^+$ and Ia$^+$ (Feldmann, Beverley, Dunkley & Kontiainen, 1975; Vadas *et al.*, 1976). In fact, the non-specific suppressor has the same Ly phenotype as the T cells that mediate help (Cantor & Boyse, 1975; Vadas *et al.*, 1976) and delayed-type hypersensitivity (DTH) reactions (Vadas *et al.*, 1976). However, whether the non-specific suppressor is actually a helper or DTH T cells remains to be established.

It appears that FCS is needed for generation of the non-specific suppressor T cells and is also required during the effector phase of the suppressors (Table 4). This result suggests that the suppressors are directed against FCS antigens and, whilst responding to these antigens, in some way non-specifically suppress humoral immunity. It seems unlikely that the suppressors are being activated by mitogens in the FCS as, if this were the case, FCS would not be required in the cultures during the effector phase of suppression. Furthermore, based on the dose response kinetics of the suppressors (Fig. 1), it seems likely that the T cells that recognize FCS antigens either directly inactivate B lymphocytes or inactivate accessory cells required for antibody formation, which have passively absorbed these antigens. In fact, this system of non-specific immunosuppression is reminiscent of the old phenomenon of 'antigenic competition', which has been observed in many *in vivo* and *in vitro* immune responses (Pross & Eidinger, 1974).

One other laboratory has also reported the spontaneous appearance in *in vitro* cultures of T cells that non-specifically suppress antibody responses (Burns, Marrack, Kappler & Janeway, 1975). Presumably these suppressors were also directed against FCS antigens present in the tissue culture medium. Furthermore, it has been demonstrated in the guinea pig (Forni & Green, 1976) and the rat (Tsutsui & Everett, 1975) that cytotoxic T cells can be generated *in vitro* against xenogeneic serum protein antigens and can subsequently kill target cells which have absorbed these serum proteins on their surface. These studies suggest that the non-specific suppressor T cell described in this paper may be a cytotoxic T cell which kills B lymphocytes or accessory cells which have acquired FCS antigens. This explanation seems unlikely, however, as cytotoxic T cells in CBA/H mice have a different Ly phenotype from the suppressor cells, i.e., they are either Ly-1$^+$, 2$^+$, Ia$^-$ or Ly-1$^-$, 2$^+$, Ia$^-$ (Shiku, Kisielow, Bean, Takahashi, Boyse, Oettgen & Old, 1975; Beverley *et al.*, 1976).

In conclusion, it should be emphasized that the appearance of non-specific suppressor T cells should be taken into account when the *in vitro* generation of T cell effectors is being attempted. It is certainly possible to generate antigen-specific helper (Kontiainen & Feldmann, 1973) and suppressor (Kontiainen & Feldmann, 1976; Eardley & Gershon, 1976) T cells *in vitro*. Presumably, in these successful studies, batches of FCS were used which minimized the development of non-specific suppressors. On the other hand, non-specific suppression can be overcome by never carrying out both primary and secondary cultures in the same type of serum.

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Announcements

4th International Symposium on Immunology of Reproduction 19–22 September 1978, Varna, Bulgaria

The Symposium, organized by the International Co-ordination Committee for Immunology of Reproduction (Sofia) and the International Society of Immunology of Reproduction (Paris), will offer broad opportunities for presentation and exchange of most recent information on the fundamental, research and clinical aspects of immunology of fertility and infertility, comprising the following problematics:

(A) *Plenary sessions:* (1) Attempts at diminishing and eliminating the immunologically conditioned infertility; (2) Mechanisms of the immune response (humoral and cellular); (3) Immunological methods for fertility regulation control.

(B) *Section sessions:* (1) Sperm and seminal plasma antigens and antibodies; (2) Ovum antigens and antibodies; (3) Fertilization as an immune phenomenon; (4) Mother response to the zygote, embryo and placenta; (5) Mother/fetus interrelations; (6) Reproductive hormones and the antibodies induced by them; (7) Immunology of lactation; (8) Modern methods in immunology of reproduction research; (9) Immunogenetic problems of reproduction.

(C) *Panel discussions*: Immunological problems of auto-, iso- and hetero-transplantation of blastocysts.

(D) *Workshops*: (1) Isolation of sperm antigens; (2) Local immunity; (3) Immunological methods for diagnostics of ovulation and pregnancy; (4) Cell-mediated immunity.

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