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Impaired primary in-vitro antibody response in progressive systemic sclerosis patients: rôle of suppressor monocytes

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SUMMARY

The primary in-vitro antibody response developed by peripheral blood mononuclear cells (PBM) towards trinitrophenyl coupled to polyacrylamide beads (TNP–PAA) was evaluated in 17 untreated patients with progressive systemic sclerosis (PSS). This response was markedly depressed as compared with that of 19 control patients and 28 normal subjects. In eight PSS patients and eight normal controls the anti-TNP response was measured before, and after, a PBM filtration on nylon wool columns. This procedure dramatically reduced the proportion of monocytes identified as mononuclear cells staining positively for peroxidases, and restored the response of PSS PBM to the level observed in normal PBM. In four experiments, plastic-adherent cells from either normal subjects or PSS patients were added to autologous nylon-passed PBM. This did not modify the response from normal PBM but inhibited the response of PSS PBM. The inhibitory effect of PSS plastic-adherent cells was insensitive to a 2,000 R X-ray irradiation. These results strongly suggest that the impaired in-vitro antibody response observed in PSS can be attributed to a suppressor monocyte. The concanavalin-A-induced suppressor cells of the antibody response were assayed in PSS. They exerted a suppressive effect to the same extent as in controls.

INTRODUCTION

Numerous abnormalities of the cellular immunity have been reported in patients with progressive systemic sclerosis (PSS). These abnormalities include depressed delayed-type hypersensitivity (Niwa & Kanoh, 1979), decreased lymphocyte response to mitogens (Horwitz & Garrett, 1977; Hugues *et al.*, 1977), impaired antibody-dependent cell-mediated cytotoxicity (Cooper *et al.*, 1978; Wright *et al.*, 1979) and decreased number of circulating T lymphocytes (Horwitz & Garrett, 1977; Gupta *et al.*, 1979) with abnormal proportions of T cells possessing receptors for IgM (Tμ) or for IgG (Tγ) (Gupta *et al.*, 1979).

In contrast, in a disease where immunoglobulin (Ig) disorders are common, studies on the ability of PSS patients to mount an antibody response are fewer and provide conflicting results (Niwa & Kanoh, 1979; Kallenberg *et al.*, 1981).

This led us to undertake the present work to investigate the ability of peripheral blood lymphocytes from untreated PSS patients to mount a primary in-vitro antibody response to trinitrophenyl–polyacrylamide beads (TNP–PAA). This model of specific in-vitro antibody

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response (Delfraissy *et al.*, 1977) has proved valuable in the study of the cellular requirements and regulation of the human B cell response (Delfraissy *et al.*, 1978; Galanaud, 1979; Galanaud *et al.*, 1979), and in some pathological conditions (Delfraissy *et al.*, 1980; Segond *et al.*, 1979). It can be used to identify the cellular basis of a defective response (Delfraissy *et al.*, 1980).

MATERIALS AND METHODS

Subjects

Patients. Thirty-six patients who had never been under immunosuppressive therapy were selected for study. None of these patients had received corticosteroid therapy in the month before testing, and aspirin or non-steroidal anti-inflammatory agents were stopped at least 72 hr before blood collection. These patients belonged to a PSS group and a control group.

(a) *PSS group.* This group consisted of 17 patients, 13 female and four male with a mean age of 52 ± 9 years (mean $\pm$ s.d.). The diagnosis of scleroderma was established by the presence of a Raynaud's phenomenon and of characteristic cutaneous lesions (in most cases biopsy-proven) involving more than just the digits. Five patients had a CREST syndrome and one a CRST syndrome (Velayos *et al.*, 1979). All patients were assessed for the extent of systemic involvement by the disease using previously described criteria (Hugues *et al.*, 1977). On this basis, patients with PSS were divided into categories of moderate (two women and two men), mild (five women and two men), or severe (five women and one man) disease.

(b) *Control patients.* Nineteen hospitalized patients, ten female and nine male, mean age 47 ± 9 years (mean $\pm$ s.d.) were studied. Twelve of these patients suffered osteoarthrosis, four ankylosing spondylitis and three a neurological disease.

Normal control population. This group was made up of 28 healthy subjects from the medical and laboratory staff, 18 female and ten male, with an age range of 16–53 years.

Lymphocyte cultures

Blood was drawn on Liquemine (Roche, Neuilly-sur-Seine) and mononuclear cells [peripheral blood mononuclear cells (PBM)] were isolated on Ficoll-Metrizoate. The primary in-vitro antibody response was induced as already described (Delfraissy *et al.*, 1977; Galanaud, 1979). PBM were cultured at a 5×10^6 ml^{-1} concentration in a culture medium containing 10% fetal bovine serum (FCS). They were incubated for 7–8 days without antigen or with TNP–PAA. Cells were recovered at the end of culture period and counted in a Coulter Counter. In some instances cell viability was assessed by the trypan blue dye exclusion test.

Assay for the anti-TNP response and for immunoglobulin-producing cells

The in-vitro antibody response was assessed by enumeration of the anti-TNP antibody-forming cells (AFC) (Delfraissy *et al.*, 1977). At the end of the culture period, cells were collected, separated from the beads, washed and used in plaque assays performed in agarose (Indubiose, Industrie Biologique Française). The target erythrocytes were both TNP-conjugated sheep red blood cells (TNP-SRBC) and native SRBC. The results are expressed as the number of direct anti-TNP AFC 10^{-6} collected cells.

The number of Ig-secreting cells was measured by a reverse plaque-forming cell (PFC) assay (Hillion *et al.*, 1980). The reverse PFC were enumerated using SRBC coated with chromium chloride with a purified anti-human $F(ab')_2$ antibody kindly supplied by Professor J. Pillot. The developer anti-Ig serum (Miles Laboratory, Elkhart, Indiana) was adsorbed with SRBC. The results are expressed as the number of PFC10^{-6} collected cells.

Mononuclear cell identification

The percentage of monocytes and of B cells among PBM was evaluated as reported by Preud'homme & Flandrin (1974). PBM were stained with a fluorescent polyspecific goat anti-human Ig serum and then for peroxidases (Pox). B cells were defined as surface-Ig-positive and

Pox-negative cells, monocytes as Pox-positive mononuclear cells. T cells were identified as cells forming E rosettes with SRBC (E^+ cells).

Cell fractionation procedures

Cells isolated in these experiments were cultured in a medium containing 5×10^{-5} M 2-mercapto-ethanol (2-ME, Schwarz-Mann, New York).

(i) *Unfractionated PBM.* These PBM were directly isolated from blood by centrifugation on Ficoll-Metrizoate as already described.

(ii) *Nylon-passed PBM.* Heparinized blood was introduced on a nylon wool column and was incubated for 45 min at 37°C (Luzzatti *et al.*, 1979). Nylon-non-adherent cells were then recovered by washing the column with warm culture medium and PBM were isolated from effluent cells by centrifugation on Ficoll-Metrizoate. This procedure resulted in approximately 50% cell loss as compared with direct isolation. In addition, nylon wool filtration modified the proportion of monocytes and lymphocytes. Indeed, in seven normal subjects, this procedure dramatically reduced the proportion of Pox-positive cells ($5{\cdot}3 \pm 0{\cdot}8\%$ *vs* $22{\cdot}5 \pm 4{\cdot}4\%$, $P < 0{\cdot}005$), moderately depleted B lymphocyte ($10{\cdot}4 \pm 1{\cdot}7\%$ *vs* $12 \pm 2\%$, $P < 0{\cdot}05$) and moderately increased the proportion of E^+ cells ($73{\cdot}7 \pm 2{\cdot}3\%$ *vs* $68{\cdot}5 \pm 2{\cdot}5\%$, $P < 0{\cdot}02$).

(iii) *Plastic adherent cells.* Five million PBM cells were incubated for 90 min at 37°C in culture dishes. Non-adherent cells were then discarded by washing the dishes with warm medium. In some experiments nylon-passed PBM were cultured in petri dishes pre-coated with plastic-adherent cells. In some instances adherent cells were recovered with a polystyrene 'policeman' and stained for characterization.

Con-A-induced suppression of the antibody response

Con A was added at a 0·5 μg ml^{-1} final concentration with the antigen, to TNP–PAA stimulated cultures. This induces a suppression of the anti-TNP response which is maximum at the peak (Galanaud *et al.*, 1979). In transfer experiments, PBM were incubated for 48 hr, without antigen, either in the presence of 2 μg ml^{-1} Con A, or without Con A for control cells. At the end of this incubation period, cells were collected, washed in the presence of α-methyl-mannoside and added as $1{\cdot}5 \times 10^6$ cells to cultures of 5×10^6 allogeneic responding cells. The latter cultures had been set up 2 days earlier and were stimulated with TNP–PAA. We have shown that, in these conditions, Con-A-stimulated PBL (or T cells) can suppress the anti-TNP response of responding cells (Galanaud *et al.*, 1979).

RESULTS

Defective antibody response in PSS patients

The in-vitro antibody response of unfractionated PBM from 17 PSS patients was compared with that of 19 hospitalized control patients and with that of 28 normal controls. The mean response of PSS PBM cultures was 24 ± 7 AFC 10^{-6} (mean $\pm$ s.e.m.), significantly depressed when compared with the response of control patients (148 ± 26 AFC 10^{-6}; $P < 0{\cdot}001$), and with that of normal individuals (167 ± 32 AFC 10^{-6}; $P < 0{\cdot}005$).

The total cell recovery at the end of the culture period was similar in all three groups, as was the percentage of viable cells ($79 \pm 3\%$; $75 \pm 4\%$; $77 \pm 3\%$, respectively).

The kinetics of response in the PSS and the control cultures were similar and the in-vitro antibody response proved reproducible in two PSS patients who were tested twice at a 2-week and a 1-month interval, respectively.

The background anti-TNP response in unstimulated cultures was the same in PSS (3 ± 1 AFC 10^{-6}), in control patients (2 ± 1 AFC 10^{-6}), and in normal controls (5 ± 1 AFC 10^{-6}). Similarly, the background anti-SRBC response in both unstimulated and TNP–PAA-stimulated cultures was low and comparable in the three groups (3 ± 1; 4 ± 1; 3 ± 1 AFC 10^{-6}, respectively).

The search for anti-TNP AFC among freshly isolated cells performed in two PSS patients and three normal controls was negative, as was the search for IgG anti-TNP AFC at the end of

stimulated culture in two PSS patients and six normal subjects. In two experiments, PBM were incubated for 18 hr in FCS-containing culture medium at 37°C. Cells were then washed extensively and cultured in the presence of TNP–PAA. Pre-incubation under these conditions did not restore an anti-TNP response to PSS PBL, nor did it modify the response of control PBM.

In contrast with this impaired anti-TNP response from PSS PBM, the amount of Ig-secreting cells at the end of the culture period was similar in seven normal controls and seven PSS patients (see Table 1).

Restoration of antibody response in PSS by nylon wool filtration

In eight experiments, we compared the in-vitro antibody response of unfractionated PBM and nylon-passed PBM (Table 2). In normal subjects, nylon filtration moderately increased the anti-TNP response. The enhancement observed in PSS was much more obvious ($P = 0{\cdot}05$) and the anti-TNP response of nylon-passed PBM was similar in patients and in normal controls. The nylon wool filtration enhanced the reverse PFC response of PBM from four PSS patients ($P < 0{\cdot}005$) and four normal controls ($P < 0{\cdot}03$). However, the reverse PFC response was comparable in both groups, before and after this filtration (Table 2). In four experiments, we evaluated the percentage of Pox-positive cells among unfractionated and nylon-passed PBM. This percentage was comparable in PSS and normal unfractionated PBM. In both groups it declined dramatically upon nylon filtration. The percentage of T lymphocytes was moderately increased and the percentage of B lymphocytes was moderately decreased among nylon-passed PBM from the same patients and controls (Table 2).

Table 1. Non specific B cell response: The number of immunoglobulin-secreting cells was evaluated by a reverse plaque assay at the end of control and TNP–PAA-stimulated culture in PSS patients and normal controls. Results are expressed as PFC 10^{-6} collected cells at the end of culture

	Number of subjects	Unstimulated cultures	TNP–PAA-stimulated cultures
PSS patients	7	1093 ± 351*	1425 ± 403*
Normal controls	7	808 ± 120	1079 ± 169

* Mean ± s.e.m.

Table 2. Nylon-passed PBM from PSS patients exert a normal anti-TNP response: The effect of nylon wool filtration of PBM from PSS patients and normal controls was assessed in four to eight experiments. Results are expressed as mean ± s.e.m.

		Nylon wool filtration of PBM from			
		PSS patients		Normal controls	
	Number of experiments	Before	After	Before	After
Anti-TNP response (AFC 10^{-6})	8	35 ± 15*	342 ± 130	135 ± 35*	405 ± 122
Ig-secreting cells (PFC 10^{-6})	4	1,156 ± 350	3,780 ± 380	1,286 ± 115	3,001 ± 499
Monocytes (%)	4	32 ± 4*	4 ± 1·2*	30·7 ± 3·4†	6·5 ± 0·5†
T lymphocytes (%)	4	57·6 ± 6·6	68·1 ± 2·4	65·9 ± 4	74 ± 4·2
B lymphocytes (%)	4	10·5 ± 1·6	8·4 ± 1·4	11·7 ± 1·7	9·8 ± 0·9

* $P < 0{\cdot}02$.
† $P < 0{\cdot}01$.

Evidence for a plastic-adherent and radio-resistant suppressor cell in PSS

In further experiments, we tested the effect of plastic-adherent cells on the anti-TNP response of nylon-passed PBM. As described earlier, 5×10^6 nylon-passed PBM were cultured in petri dishes coated with autologous plastic-adherent cells. As shown in Table 3 nylon-passed PBM from PSS patients and normal controls responded similarly. The anti-TNP response of nylon-passed PBM from normal controls was not modified by co-culture with autologous plastic-adherent cells. In contrast, nylon-passed PBM from PSS patients when co-cultured with autologous plastic-adherent cells exhibited a depressed anti-TNP response. In three experiments, four petri dishes were used to enumerate the number of coated adherent cells. This number was similar in controls and PSS ($0{\cdot}9$–$1{\cdot}3 \times 10^6$ adherent cells recovered from 5×10^6 unfractionated PBM). In addition, the proportion of Pox-positive cells was identical in both groups: $92 \pm 1\%$ in PSS as compared with $89 \pm 2\%$ in normal controls. In experiments 2–4 (see Table 3), X-ray irradiation (2,000 R) did not affect the suppressive effect of PSS plastic-adherent cells. The response of nylon-passed PBM co-cultured with irradiated autologous adherent cells was 77 ± 32 AFC 10^{-6}, as compared with 73 ± 27 AFC 10^{-6} when co-cultured with non-irradiated adherent cells.

Con-A-induced suppression of the in-vitro antibody response in PSS patients

Con A at a $0{\cdot}5$ $\mu g\ ml^{-1}$ final concentration was added at the initiation of TNP–PAA-stimulated culture in order to induce non-specific T cell suppression of the antibody response. As shown in Table 4, Con A significantly reduced the anti-TNP response of PBL from 18 normal subjects ($P < 0{\cdot}01$) and from 16 control patients ($P < 0{\cdot}005$). Similarly, in the ten PSS patients able to mount a detectable anti-TNP response, a significant inhibition was observed in the presence of Con A

Table 3. Plastic-adherent cells from PSS patients suppress the anti-TNP response of autologous nylon-passed PBM: In four experiments, nylon-passed PBM from PSS patients and from normal controls were cultured either alone, or with autologous plastic-adherent cells. The anti-TNP response (AFC 10^{-6}) evaluated at the end of triplicate cultures stimulated with TNP–PAA is provided

Experiment number	Autologous plastic-adherent cells addition in			
	PSS patients		Normal controls	
	−	+	−	+
1	149	65	339	337
2	435	65	391	309
3	94	30	134	103
4	1193	124	1152	555
Mean ± s.e.m.	468 ± 253	71 ± 19*	504 ± 223	326 ± 92*

* $P < 0{\cdot}05$.

Table 4. Suppressive effect of Con A: The anti-TNP response of PBM cultured in the absence or in the presence of $0{\cdot}5$ $\mu g\ ml^{-1}$ Con A was evaluated in controls and PSS patients. Results are expressed as the mean anti-TNP response (mean ± s.e.m.) in PBM cultures from PSS patients and controls

	Number of subjects	Anti-TNP response (AFC 10^{-6})		Suppression (%)
		Without Con A	With Con A	
PSS patients	10	32 ± 10	13 ± 5	64 ± 7
Normal controls	18	144 ± 36	41 ± 14	75 ± 4
Control patients	16	155 ± 30	45 ± 15	72 ± 7

Table 5. Con-A-incubated cells from PSS patients suppress the anti-TNP response of normal allogeneic PBM: In five experiments, PBM from a PSS patient and from a normal control were cultured for 2 days without addition (control cells) or in the presence of 2 $\mu g\ ml^{-1}$ Con A (Con-A-incubated cells). These cells were then added to TNP–PAA-stimulated cultures of a normal allogeneic donor. Results are the mean anti-TNP response in these latter cultures, on day 8 (mean ± s.e.m.)

Allogeneic cells added	Anti-TNP response in normal PBM cultures (AFC 10^{-6})		Suppression (%)
	Control	Con-A-incubated	
PSS	72 ± 21*	13 ± 5*	78 ± 9*
Normal	67 ± 20	12 ± 3	81 ± 5

* Mean ± s.e.m.

(13 ± 5 AFC 10^{-6} *vs* 32 ± 10 AFC 10^{-6} in control cultures, $P < 0{\cdot}02$). The mean suppression index was similar in PSS (64 ± 7%) and both control groups (75 ± 4% and 72 ± 7%, respectively).

Con-A-activated PSS PBL suppression of the response of normal allogeneic PBL

As the effect of Con A could not be evaluated in PSS patients with too low an anti-TNP response, cell transfer experiments were performed as detailed earlier. PBM from five PSS patients and five normal controls were incubated for 2 days either without Con A (control cells) or in the presence of Con A (Con A cells) and were then added to responding cultures of an allogeneic normal subject. As compared with the response of secondary cultures in which control cells were added, the anti-TNP response of normal allogeneic PBM was significantly suppressed upon addition of Con A cells from either PSS patients ($P < 0{\cdot}05$) or normal subjects ($P < 0{\cdot}05$) (see Table 5). The mean suppression index developed by PSS PBM was 78 ± 9% as compared with 81 ± 5% for normal PBM.

DISCUSSION

This study shows that PBM from 17 PSS patients were unable to develop a primary in-vitro antibody response as compared with that of 19 hospitalized control patients and 28 normal subjects. It should be stressed that none of the patients received corticosteroids or immunosuppressive drugs, and that anti-inflammatory agents were stopped at least 72 hr before blood collection in both the PSS and control patients. This low antibody response is not due to a decreased survival of PSS PBM in culture or to a modification in the characteristics of the anti-TNP response.

In our model, the specific antibody response is associated with the development of a non-specific B cell response detected by a reverse PFC assay (Hillion *et al.*, 1980). This non-specific response is normal in PSS thus suggesting that the defect of in-vitro humoral immunity selectively affects the ability to produce specific antibodies in a primary response. Conflicting results on the in-vivo antibody response in PSS patients have been reported (Niwa & Kanoh, 1979; Kallenberg *et al.*, 1981). Our in-vitro results are in the same line as those of Niwa & Kanoh (1979) indicating a defective humoral response in PSS patients.

The most evident abnormalities of the immune status in PSS concern cellular immunity. Delayed-type hypersensitivity (Niwa & Kanoh, 1979) and the in-vitro T cell proliferative response to various mitogens (Horwitz & Garrett, 1977; Hugues *et al.*, 1977) are depressed. These abnormalities are associated with a decrease in the number of circulating T cells (Horwitz & Garrett, 1977; Gupta *et al.*, 1979).

Our study shows a moderate decrease in the number of circulating T cells in PSS and provides information on two aspects of T cell function: (i) the Con-A-induced suppressor cells (Galanaud *et al.*, 1979) appear functionally normal; and (ii) the helper T cell function is reflected in the level of the

T-dependent anti-TNP reponse (Delfraissy *et al.*, 1977; Galanaud, 1979). Although this response is depressed, the fact that removal of nylon-adherent cells increases the anti-TNP response up to a normal level strongly suggests that helper T cell are intrinsically normal.

Indeed, the most striking result in this study is the demonstration that nylon-passed PBM from PSS patients display a normal anti-TNP response. This led us to search for a suppressor cell which could have been removed by nylon wool filtration. As this procedure dramatically reduces the proportion of peroxidase-positive mononuclear cells we looked for suppressive monocytes among PSS PBM. Monocytes were isolated by plastic adherence and this procedure yielded comparable numbers of cells (90% Pox-positive) in PSS and controls. Upon addition to autologous nylon-passed PBM, only PSS adherent cells suppress their anti-TNP response. This suppressive effect is not abolished by X-ray irradiation of adherent cells, which further suggests the monocyte nature of this suppressor cell.

To our knowledge, this is the first report of suppressor monocytes in PSS. They explain entirely the defect of the in-vitro antibody response we evidenced and they may account for previous results demonstrating an impaired cellular immunity in PSS.

Monocytes are able to suppress the in-vitro responses of lymphocytes from normal subjects (Gmelig-Meyling & Waldmann, 1981; Montazeri *et al.*, 1980) and from patients with diverse pathological conditions (Katz & Fauci, 1978; Goodwin *et al.*, 1977; Markenson *et al.*, 1978). In most instances (reviewed in Goodwin & Webb, 1980), but not in all (Gmelig-Meyling & Waldmann, 1981), this suppression is mediated by prostaglandins. It will be of importance to determine whether similar abnormalities exist in PSS. Our demonstration of a defective in-vitro antibody response which is due to a suppressor monocyte provides an approach to this question. Nevertheless, a more precise knowledge of the rôle played by these mediators in PSS could be of interest in the general understanding and management of this disease.

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