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Lymphocyte function in anergic patients

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SUMMARY

The lymphocyte function of anergic surgical patients who are at increased risk for sepsis and mortality was studied. *In vitro* lymphocyte responses appear to be normal in most instances, in that over 80% of patients showed a normal response in a standardized mixed leucocyte culture reaction. Similarly, 56% of the lymphocytes from anergic patients showed a positive *in vitro* proliferative response with PPD. The ability of *in vitro*-activated lymphocytes to elicit a skin reaction was determined by culturing the cells of anergic patients with PPD and then reinjecting the lymphocytes or their supernatants intradermally into the original donor. When there was a positive proliferative response to PPD *in vitro*, the reinjected cells or supernatant elicited a positive skin reaction in 79% of the anergic patients. In contrast, a skin reaction was obtained in less than 20% of the instances when there was no *in vitro* proliferation to PPD or when the cells were cultured without antigen. These results suggest that one of the acquired immune defects in these anergic patients is an *in vivo* block of lymphocyte activation.

INTRODUCTION

In both preoperative and postoperative studies, it has been shown that the absence of a delayed-type hypersensitivity (DTH) reaction to recall skin test antigens (i.e. anergy) in surgical patients is predictive of increased sepsis and mortality (Pietsch, Meakins & MacLean, 1977; Meakins *et al.*, 1977). Although DTH is a measure of cell-mediated immunity, infections in these patients are most often due to common Gram-positive and Gram-negative bacteria which are handled by the humoral immune and phagocytic systems (Meakins *et al.*, 1977). Accordingly, the immune defects in anergic patients are likely to encompass several if not all facets of the immune response. The present study was undertaken to investigate the cause of the lack of a DTH reaction, as well as to seek an explanation for the broader aspects of the failure of the immune response in these patients.

In the DTH reaction, lymphocytes are activated by antigen to undergo proliferation and elaboration of lymphokines. These factors in turn attract and activate the non-specific effector cells which mediate the reaction (Waksman, 1979). In the first series of experiments, we studied the *in vitro* activation of lymphocytes from anergic patients. In the second part of the study, the ability of *in vitro* activated cells and/or their products to elicit a DTH reaction in anergic patients was examined. The results obtained suggest that anergy is due in part to a defect in lymphocyte activation *in vivo*.

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MATERIALS AND METHODS

Patient population. Surgical patients, except for those taking steroids, on the surgical wards and in the intensive care unit of the Royal Victoria Hospital were studied. This study was approved by the Medical Ethics Committee of the Faculty of Medicine, McGill University and the Royal Victoria Hospital, and informed consent was obtained from each patient. The patients were skin tested by the intradermal injection of five recall skin test antigens (Candida, PPD, mumps, trichophyton and varidase) as previously described (Meakins *et al.*, 1977). Indurations of greater than 5 mm at 24 or 48 hr were considered a positive response. The patients were classified as 'normal patients' if they responded to two or more antigens, as relatively anergic if there was a response to one, and anergic if there was no response to any antigen. All the skin tests were administered and read by one person.

Experimental protocol. In an initial series of experiments using a standardized mixed lymphocyte culture (MLC) reaction (Osoba & Falk, 1974), the proliferative response of lymphocytes from anergic patients was compared to that of normal patients and of healthy volunteers. Because the results indicated normal lymphoproliferative reactivity in most individuals, the ability of *in vitro*-activated lymphocytes to initiate a DTH reaction was determined in the second part of this study. Lymphocytes of anergic patients were cultured *in vitro* with PPD. After 3 or 4 days, the cells recovered from these cultures, as well as the cell-free supernatant, were reinjected intradermally in the original cell donor. The patients were skin tested with all the test antigens at the same time, and those who responded were excluded. The skin reactions obtained by the *in vitro*-activated cells were evaluated in parallel to their proliferative response to PPD *in vitro.*

Cell preparation and culture. Heparinized blood (30–40 ml) was obtained by venepuncture and 1 ml of carbonyl iron (Technicon, Tarrytown, New York) was added. After incubation for 1 hr at 37°C, the blood was layered over Ficoll–Hypaque (Isolymph, BDH, Montreal, Quebec), centrifuged, and the lymphocytes at the interface were collected as described by Böyum (1968). The cells were washed three times with Hanks' balanced salt solution (HBSS) and then suspended at a concentration of 1×10^6 lymphocytes/ml HS medium (RPMI 1640 supplemented with 15% AB serum, 1 mM HEPES, and antibiotics–ampicillin 100 μg/ml, cloxacillin 50 μg/ml, penicillin 100 iu/ml).

The cells were cultured at 37°C in a humid atmosphere of 95% air/5% CO_2 for the desired length of time. In order to measure the proliferative response, 1 μCi of ^{3}H-thymidine (sp. act. 20 Ci/mmol, NEN, Boston, Massachusetts) was added for the last 5 hr of incubation and the reaction stopped by freezing. After thawing, the cultures were harvested with a MASH II harvester and the samples counted in a scintillation counter (Packard Instruments, Chicago, Illinois).

MLC reaction. The method of Osoba & Falk (1974) was used for standardizing the MLC reaction by utilizing a pool of stimulating cells from three individuals selected to express a total of 11 different HLA-A and B antigens. The stimulating cells were cryopreserved separately and thawed (Mangi & Mardiney, 1970), irradiated and mixed in equal numbers just before use. The cells were more than 90% viable as determined by trypan blue exclusion. The MLC cultures were set up in quadruplicate in microtest plates (Falcon 3040) using 1×10^5 responding cells and 2 and 4×10^5 stimulating cells in 0·2 ml HS medium. ^{3}H-thymidine uptake was measured on the 7th day of culture.

Activation with PPD. In order to determine if the *in vitro*-activated cells of anergic patients could elicit a skin test reaction, the cells were isolated and cultured at a concentration of 1×10^6 lymphocytes/ml of RPMI-HS medium containing 40 μg/ml PPD (Connaught Lab., Toronto, Ontario). The volume of the cultures was 10 ml (Falcon flask 3012) or 15 ml (Corning flask 25100). After incubation for 3 or occasionally 4 days, the cells were collected by centrifugation and washed; 5×10^6 lymphocytes were suspended in 0·1 ml saline and then injected intradermally. At a second site, 0·1 ml of cell-free culture supernatant was injected. Control cells injected were either cultured without PPD or were heat-killed prior to injection.

For measuring proliferative activity, an aliquot of the same cells was cultured in microtest plates using 1×10^5 cells/well in 0·2 ml RPMI-HS medium containing 40 μg/ml PPD. The proliferative response was assessed on day 7.

Biopsy. Biopsies were taken using a skin punch. The tissues were processed and the reactions were evaluated by Dr R. Michel of the Department of Pathology of the Royal Victoria Hospital.

Calculation of results. For the MLC response, two normal volunteers were always included with each group of patients tested. Two stimulating cell concentrations (0·2 and 0·4 × 10^6 per well) were always used for each test. The mean normal response was taken as the average c.p.m. of the ^{3}H-thymidine incorporated in the MLC reaction of the two normal volunteers. The lymphocyte function of patients was considered abnormal if the response for both stimulating cell concentrations lay outside the mean by ± 2 standard deviations. In these experiments, the mean response was 50,800 c.p.m. with a standard deviation of $\pm 18{,}200$.

For activation by PPD, the proliferative response was considered positive if there was a net increment of more than 5,000 c.p.m. ^{3}H-thymidine incorporated and a stimulation index greater than 5.

The skin reaction obtained by adoptive transfer of cells was evaluated by the criteria used for skin testing by antigens: a reaction was considered positive if it gave rise to an induration of 5 mm or greater, 24–48 hr after injection.

RESULTS

MLC response

The proliferative response in the MLC of anergic patients is compared to that of normal volunteers and normal patients in Fig. 1. Of the 24 normal volunteers tested, all responded within the normal range except one who had a low response. One of the 10 normal patients had a high response. Lymphocytes of 39 anergic patients were tested in 54 separate MLC reactions: with the exception of six responses which were low, and four which were high, the majority of the responses were normal. The responses of three patients who were tested sequentially are also shown in Fig. 1. One patient had an abnormally low response in the 1st week but slowly regained responsiveness during the subsequent 4 weeks. The other two patients had normal reactions throughout.

Proliferative response to PPD

The 43 anergic patients studied were divided into responsive or unresponsive groups according to the *in vitro* proliferative reaction elicited by PPD. Table 1 shows that 24 of 43 patients (56%) were

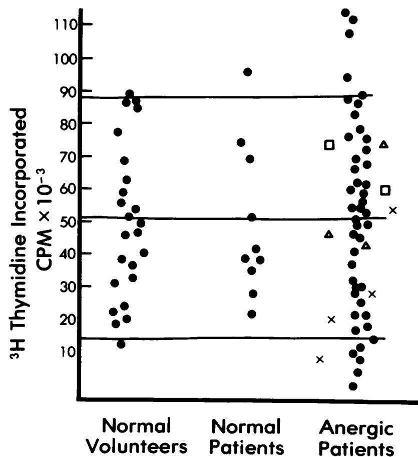

Fig. 1. Comparison of the proliferative response in the MLC of anergic patients to that of normal volunteers and normal patients. Each point represents the mean c.p.m. of ^{3}H-thymidine incorporated in the MLC reaction with 0·2 and 0·4 × 10^5 stimulating cells. The horizontal lines represent the mean c.p.m. ± 2 s.d. of the MLC response of lymphocytes of normal volunteers. The symbols (×, ▵, ▫) refer to individual MLC responses at weekly intervals of cells from three different anergic patients.

Table 1. Skin reactivity of anergic patients

No. of patients	Patient status (*in vitro*)†	Cells cultured	No. of patients injected	Mean c.p.m. ± s.e. incorporated	Skin response elicited by: Supernatant from cultured cells	Living cells in NaCl
19	Unresponsive	No PPD	10	1,072 ± 356	0/8	2/9
		PPD	19	3,003 ± 839	2/16	3/17*
24	Responsive	No PPD	13	1,039 ± 277	0/10	2/12
		PPD	24	21,071 ± 3,535	11/19	15/24*

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

† All the patients studied were anergic, i.e. unresponsive *in vivo*; the responding status cited in this column refers to their reactivity to PPD *in vitro*.

responsive to PPD *in vitro*. As this figure is comparable to the proportion of healthy individuals in this hospital who are sensitive to PPD by skin testing (Christou, Meakins & MacLean, 1981), it is reasonable to assume that lymphocytes of PPD-sensitive anergic patients do respond to PPD *in vitro*, and those who do not respond fail to do so through a lack of previous exposure to this antigen.

Skin reactions elicited by cells activated in vitro

The lymphocytes of anergic patients were cultured *in vitro* with or without PPD and then an aliquot of the cells or supernatant was reinjected intradermally into the original donor to determine their ability to elicit a skin reaction. An aliquot of the same cells was further cultured to determine the *in vitro* proliferative responsiveness to PPD. When heat-killed cells were injected, a positive skin reaction was obtained in only one of 26 instances (results not shown). When the cells and supernatants injected were derived from control cultures containing no antigen, only two of nine and two of 12 of the cell preparations, and none of eight and none of 10 of the supernatants elicited a skin reaction, regardless of the ability of the cells to respond to PPD *in vitro* (Table 1). In contrast, when the cells or supernatants from cultures incubated with PPD were injected, cells from patients which responded to antigen *in vitro* elicited a positive skin reaction with a frequency of 15 of 24 with

Table 2. Skin reactions elicited in anergic patients with cultured cells and supernatants*

	Unstimulated‡	Stimulated
Unresponsive†	2/10 (20)	3/19 (15·7)
Responsive†	2/13 (15·3)	19/24 (79·2)

* The numbers recorded represent the sum of positive reactions to the injection of cultured cells or cell-free supernatants in each patient.

† All the patients studied were anergic, i.e. unresponsive *in vivo*; the responding status cited in this column refers to their reactivity to PPD *in vitro*.

‡ Stimulated refers to cultures with, unstimulated to cultures without antigen. Figures in parentheses express results as a percentage.

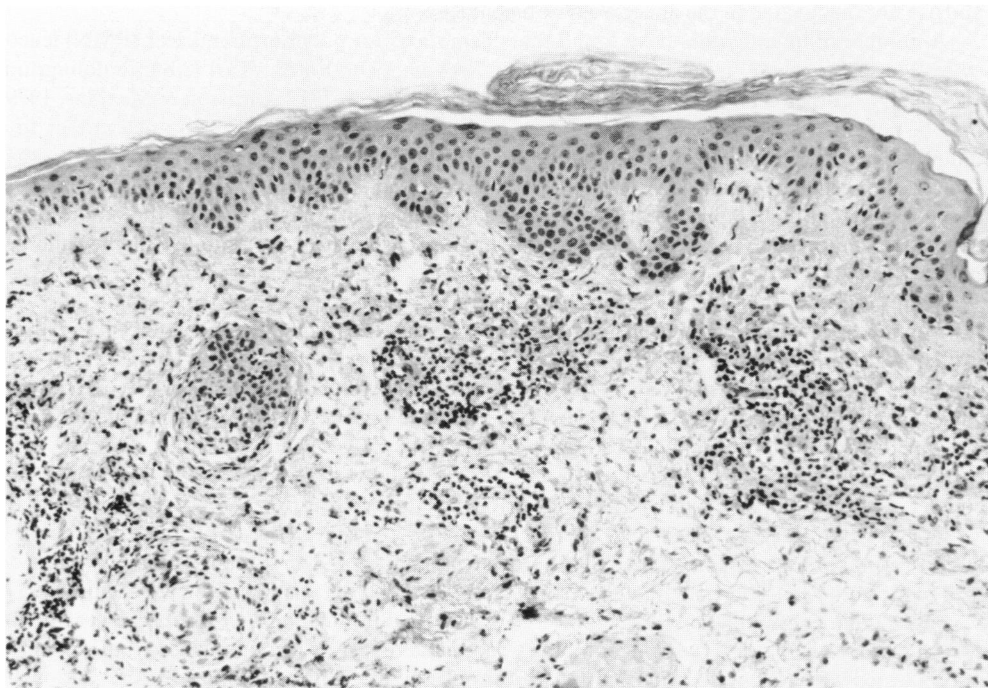

Fig. 2. Biopsy of the skin reaction obtained at 24 hr after the intradermal injection of the culture supernatant of *in vitro* PPD-activated lymphocytes. (Original ×40.)

cells and 11 of 19 with supernatant, whereas those from patients whose cells did not respond *in vitro* gave a positive response in two of 16 instances with cells and three of 17 with supernatants.

A summary of these results is presented in Table 2, showing the total number of patients in whom skin reactions were elicited by cells or supernatant. In the group of anergic patients whose lymphocytes did not respond to PPD *in vitro*, a positive skin reaction was obtained in less than 20% of the instances when either cells or supernatants were reinjected. However, in the group of anergic patients whose cells were cultured with and responded to antigen *in vitro*, a positive skin reaction was obtained upon reinjection of cells or supernatant with a frequency of over 79%.

Skin biopsies

A biopsy was taken of the skin reaction elicited by PPD from an anergic patient who became reactive during the experiment. Biopsies were also taken of the reaction elicited by *in vitro*-activated cells and by supernatant in an anergic patient. All the skin reactions obtained had a similar morphology with a predominantly mononuclear cell infiltrate (Fig. 2).

DISCUSSION

The results suggest that the lymphocytes in the majority of anergic patients had normal lymphoproliferative capability *in vitro*. This is shown not only by the MLC reaction, but also by the positive responses elicited by PPD. Over 55% of anergic patients responded to PPD *in vitro*.

The ability of the lymphocytes (or their supernatants) after activation *in vitro* to elicit a skin response when reinjected into anergic patients also demonstrates the normal function of these cells, which includes attraction and activation of the non-specific effector cells to the injection site which are responsible for the typical DTH reaction (Waksman, 1979). Accordingly, it would appear that the defect in at least 75% of these patients lies at the activation step and not in the attraction of cells

to the skin test site. Once the cells are activated they also appear capable of fulfilling their function and are not inactivated in the anergic environment.

A number of investigators have found that chemotaxis for polymorphonuclear (PMN) leucocytes (Pietsch *et al.*, 1977; Meakins *et al.*, 1977; Grogan, 1976; Fikrig, Karl & Sunthalolingrum, 1977) and for other cell types (Altman, Furrkawa & Klebanoff, 1977; Christou & Meakins, 1979) may be defective in anergic and burn patients and these writers have thus attributed anergy to a failure of effector cells to reach the skin test site. However, the results described here suggest that such effector cells can usually reach the reaction site provided the necessary mediators are available. It is possible that the lymphocytes (i.e. the source of mediators) are not reaching the skin test site, although a more likely explanation is that lymphocyte activation is inhibited in the 'anergic environment'.

There could be a number of different ways in which lymphocyte activation is inhibited *in vivo*. Macrophages, essential for lymphocyte activation, have been claimed to be both hyporeactive (Miller, Graziano & Lim, 1979) or alternatively hyper-reactive (Keller, 1975; Metzger, Hoffeld & Oppenheim, 1980) in disease states. Whereas hyporeactivity would not seem to explain the normal *in vitro* lymphocyte reactivity, inhibition by hyper-reactive or excess macrophages cannot be ruled out, since the majority of phagocytes were removed from our patients' blood by treatment with carbonyl iron. Lymphocyte activation could also be inhibited by excessive suppressor T-cell activity, which would have to be confined to *in vivo* conditions. However, this possibility is unlikely as such cells are readily detected *in vitro* (Naor, 1979). Another source of inhibition may be inhibitory factors present in the circulation of anergic patients which inhibit both lymphocyte proliferation to PHA (McLoughlin *et al.*, 1979) and PMN chemotaxis (Christou & Meakins, 1979; Van Epps, Palmer & Williams, 1974; Smith *et al.*, 1972). In our initial report (MacLean *et al.*, 1975), two patients studied had very strong inhibitory serum factors. However, in this study, the majority of sera tested were only weakly inhibitory: only about 15% of the sera inhibited the MLC response by more than 75% and few gave complete inhibition at the concentrations used. Nevertheless, we have recently found that the majority of sera inhibit the response of lymphocytes to PPD (manuscript in preparation), and it is possible that such factors will prevent lymphocyte activation.

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