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Role of cytokines in the restoration of the delayed-type hypersensitivity reaction of anergic patients

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SUMMARY

Soluble mediators from peripheral blood lymphocytes activated either by skin test antigens or by alloantigens restored the delayed type hypersensitivity (DTH) reaction in the majority of anergic surgical patients who are at increased risk for sepsis and mortality. Antigen had to be injected together with the mediators and the individual had to be reactive to the antigen for restoration. These results suggest that restoration of the DTH response depends on the ability of cytokines produced and acting in a non-specific manner to promote the response of the anergic patients' specific antigen-sensitive cells to antigen.

Keywords Delayed-type hypersensitivity lymphokine anergy cytokine

INTRODUCTION

Surgical patients who have an absence of delayed-type hypersensitivity (DTH) reactions to recall skin test antigens (i.e. are anergic), have a high incidence of sepsis and mortality (reviewed by Christou, 1985). We have previously shown that the anergic state is characterized by an *in vivo* block of lymphocyte activation rather than an absence of functional cells. Thus not only could peripheral blood lymphocytes (PBL) of anergic patients be activated *in vitro* with skin test antigens but these activated cells when reinjected intradermally into the original donor could elicit a skin reaction. Furthermore, the culture supernatant of such activated cells were able to restore the local DTH reaction which we attributed to the presence of soluble mediators released from the activated cells (Rode *et al.*, 1982).

In the DTH reaction there is accumulation with time of mononuclear cells at the site of antigen injection. This accumulation is the result of sensitized lymphocytes recognizing antigen in the context of the major histocompatibility complex (MHC) and releasing chemoattractants which in turn recruit and activate the non-specific mononuclear bone-marrow derived effector cells which constitute the classic infiltrate of the DTH reaction (Askanase & Van Loveren, 1983; Waksman, 1979). The restoration of the DTH reaction in the anergic state by soluble mediators indicates that the non-specific effector cells can be attracted to the reaction site and therefore this component of the DTH reaction is intact. However the identity and thus the role of the factor(s) in restoring the response is unknown: the active component could simply be a non-specific chemoattractant released from the sensitized lymphocytes activated with antigen (Dwyer & Kantor, 1973; Suko, Yoshida & Cohen, 1985) or alternatively it may be one contributing to the activation of sensitized cells (Palacios, 1982). The experiments reported in this paper were initiated to analyse the role played by the mediators and thus further define the defect in anergy.

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MATERIALS AND METHODS

Patient population

Forty-three individuals admitted for treatment of surgical conditions to the surgical wards or in the surgical intensive care unit of the Royal Victoria Hospital, referred to as 'surgical patients' were studied. Those under chemotherapy or radiotherapy or who had been given corticosteroids were excluded from this investigation. 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 described by Meakins *et al.* (1977). Indurations of greater than 5 mm at 24 or 48 h were considered a positive response. The patients were classified as 'reactive' if they responded to two or more antigens or as 'anergic' if there was no response to antigen. All the skin tests were administered and read by one person. The 43 patients entered in this study were all anergic. They were divided into two groups on the basis of the *in vitro* proliferative response of their lymphocytes to one of the skin test antigens (PPD) taken as an indicator of their previous exposure to this antigen. The

individuals in the two groups were comparable in their age (mean of 69 vs 68 years) and severity of their illness as judged by their acute physiological score (mean APS 7 versus 7·7, with seven patients in each group with a score above 10).

Experimental protocol

Two standard mediator-rich supernatants were generated from peripheral blood leucocytes of reactive donors and were used throughout. One was raised in a 3 day culture against PPD (referred to as allogeneic supernatant containing antigen), and the other against allogeneic cells (called antigen-free supernatant or MLC). The third type of supernatant used was from PBL of anergic patients cultured with PPD for 3 days; aliquots of these cells were also cultured for 7 days to evaluate their proliferative response to PPD. All supernatants harvested were cleared by centrifugation and sterilized by millipore filtration. The standards were checked for hepatitis and HTLV-III virus and bacterial contamination before injection; the antigen-free supernatant was also concentrated 5-fold on an Amicon membrane with a 1,000 dalton pore size.

The test subjects were injected intradermally with 0·1 ml of antigen alone, MLC supernatant alone and culture supernatant adjusted to contain the equivalent amount of antigen. The skin reactions were read at 12, 24 and 48 h.

Cell preparation and culture

Heparinized blood was drawn by venepuncture, mixed with an equal volume of Hank's Balanced Salt Solution (HBSS) layered over Ficol-Hypaque (Ficoll-Paque, Pharmacia, Montreal, Quebec), centrifuged and the lymphocytes at the interface collected as described by Böyum (1968). The cells were washed three times with HBSS and then resuspended in HS medium (RPMI 1640 supplemented with 10% AB human serum). The HBSS and RPMI 1640 were obtained from Gibco, Burlington, Ontario. For the generation of the standardized mediator-rich supernatant the cells were cultured at 1×10^6 cells/ml with 40 μg/ml of PPD (Connaught Labs, Toronto, Ontario) or as an equal mixture of cells from two unrelated donors in 15 ml (Corning flask 25100) or 80 ml (Falcon flask 3024) of HS medium, while the PBL of anergic patients were cultured under the same conditions in 2 ml (Falcon tube 2027). For measuring proliferative activity, a sample of the same cells was cultured in microtest plates (Falcon 3070) using 1×10^5 cells/well in 0·2 ml HS medium. 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, MA) was added for the last 5 h 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, IL).

Anergic patients were categorized as responsive or unresponsive to PPD on the basis of the proliferation of their PBL to PPD; the proliferative response was considered positive if there was a net increment of more than 4000 ct/min ^{3}H-thymidine incorporated and a stimulation index greater than 5.

The skin reactions obtained following injection of the mediators were read blind and evaluated by the same criteria as used for the skin test antigens.

Table 1. DTH* reactions elicited by the injection of cell-free supernatants derived from lymphocytes cultured with PPD

	Preparation injected	
Patients skin-tested†	Autochthonous§ Supernatant +PPD	Allogeneic§ Supernatant +PPD
Anergic, responsive to PPD *in vitro* 11477±743+PPD‡ 659±612 control	10/16	9/17
	($P<0·01$)	($P<0·02$)
Anergic, unresponsive to PPD *in vitro* 1525±1277+PPD‡ 310±289 control	1/15	1/15

* DTH reactions were read at 24 h.

† The anergic patients skin tested were divided into two groups on the basis of the *in vitro* proliferative response of their lymphocytes to PPD as indicated; neither group was reactive to PPD *in vivo*.

‡ Mean ct/min ^{3}H-thymidine incorporated when the cells were cultured with or without PPD (control).

§ The supernatants injected were the patients' own (autochthonous) and one standard preparation (allogeneic) derived from cells of a PPD reactive healthy donor; each supernatant contained PPD.

Biopsies

Biopsies were taken using a skin punch, immediately frozen and processed under the supervision of Dr S. Jothy, Department of Pathology, Royal Victoria Hospital. Skin sections were investigated using immunoenzymatic staining procedures modified from Warnke & Levy (1980). The monoclonal antibodies used were OKT11, 4, 8, M-1 and B7 (Ortho Pharmaceuticals, Don Mills, Ontario). The sections were incubated with the monoclonal antibody, then with rabbit anti-mouse Ig followed by swine anti-rabbit Ig peroxidase conjugated and finally with the substrate Diaminobenzidine and H_2O_2. The sections were counterstained with haematoxylin and evaluated using light microscopy.

RESULTS

We have previously shown that the majority of anergic patients judged to have been sensitized to PPD on the basis of the proliferative response of their lymphocytes to PPD, would give a DTH response when injected with PPD together with a culture supernatant from their PPD activated cells. In contrast such a procedure failed to incite a response in patients whose cells did not react to PPD *in vitro*. Experiments confirming these observations are shown in Table 1. Failure to elicit a DTH reaction in the second group may have been due to a paucity of lymphokines generated *in vitro* by their cells or to a lack of PPD-responsive cells *in vivo* or to both. To assess unequivocally the *in vivo* PPD-reactive status of these patients, we injected them with PPD and a PPD generated supernatant from a healthy PPD-reactive donor. Although this supernatant restored the DTH reaction in nine of 17 PPD reactive patients, it failed to facilitate

Table 2. DTH reactions obtained with MLC supernatants

Subjects skin-tested	Preparation injected		
	MLC	PPD*	MLC+PPD*
Anergic patients responsive to PPD *in vitro*	0/25	0/25	18/25
Healthy individuals non-sensitive to Ag*	0/10	0/10	2/10

* These individuals have not been previously exposed to the antigen as assessed by their failure to give a DTH reaction *in vivo*. The antigen used in two subjects was not PPD but trichophyton.

a response in 14 of 15 PPD unresponsive individuals (Table 1). These observations implied that the DTH response of anergic patients to antigen and culture supernatant depended on the presence of PPD responsive cells in these individuals and thus imputed an antigen-dependence and antigen specificity to the skin reaction. The following experiments were designed to prove this specificity and probe its nature.

To assess the requirement for antigen and the specificity of the mediator, a supernatant free of skin test antigen was generated in mixed leucocyte cultures of cells from healthy donors (MLC). Such preparations injected alone into the skin of anergic patients (reactive to PPD *in vitro*) did not give a skin reaction, nor did PPD alone. However when injected together a DTH-like skin reaction was seen at 24 h in 18/25 patients (Table 2), hence with a frequency comparable to that obtained with mediators raised with the specific antigen PPD. The same MLC supernatant was also injected into 10 healthy individuals by itself, or together with a skin test antigen to which these individuals had no prior sensitivity: in the majority of the subjects (8/10), the supernatant did not confer reactivity against the irrelevant antigen (Table 2).

Skin biopsies

Previously we have shown that antigen and supernatant from activated cells of PPD reactive individuals gave a skin reaction with a mononuclear cellular infiltrate similar in appearance to a normal DTH reaction. In order to assess the reactions obtained with MLC supernatant, skin biopsies were taken from skin sites of anergic individuals injected with PPD alone, MLC alone or MLC plus PPD after 24 h. Biopsies were also taken both from normal reactive and from anergic individuals injected with MLC supernatants 12 and 24 h earlier. The reason to include MLC 12 and 24 h reactions was that this preparation elicited a response with erythema and oedema 10–14 h after injection, which however disappeared by 20 h. At least two biopsies were taken of each type of injection site. Antigen alone (Fig. 1a) or the MLC supernatant alone at either 12 (Fig. 1c) or 24 h (Fig. 1d) attracted few cells to the injection sites, while a typical rich mononuclear infiltrate was seen when antigen and supernatant were injected together (Fig. 1b). The cells at the site were predominantly T lymphocytes and macrophages; few B cells were found in the infiltrate. The T lymphocytes were a mixture of both helper and suppressor/cytotoxic cells as detected by anti-T4 and anti-T8 antibodies (not shown).

DISCUSSION

The results described confirm our previous observation that restoration of the DTH response of anergic patients to PPD by the injection of culture supernatants is dependent on their previous exposure to the antigen as attested by a proliferative reaction of their lymphocytes to PPD *in vitro*. We have now shown that this restored response is antigen-dependent, antigen-specific, and that this specificity resides with the host's cells reacting at the skin site and not with the injected mediators. This has been accomplished through the use of supernatants raised in MLC, which, unlike the preparations previously used, were free of PPD. Injection of the MLC-derived mediators which together with antigen yielded a DTH reaction in PPD-reactive anergic patients were ineffective in eliciting a response in the same individuals when injected alone, without antigen. Furthermore, cytokines and antigen combined gave no reaction in PPD-unresponsive patients. Accordingly we conclude that the DTH response in anergic patients is a reaction of antigen-specific cells in response to antigen which however requires assistance by the injected cytokines.

Our previous study revealed that for activity *in vivo*, culture supernatants had to be derived from activated cells, but provided no information as to the specificity of the cytokines. The present experiments disclosed that the antigen used *in vitro* to raise the cytokines and that used in the skin test did not need to be the same, demonstrating that both the elicitation and the mode of action of the factors was antigenically non-specific, and accordingly the entire specificity of the DTH reaction resided with the host's cells *in situ*. The same experiments indicated that the MLC-derived cytokine(s) injected alone, i.e. without PPD, did not elicit a DTH reaction in anergic patients, and furthermore they were also without effect when injected with an inappropriate antigen to healthy reactive individuals. These observations distinguish the MLC-derived mediator(s) from the late acting skin reactive factor (Dwyer & Kantor, 1973; Suko *et al.*, 1985) which requires no antigen. The MLC supernatant did give an oedematous response in most individuals at 12 h which waned by 20 h, and hence could be distinguished from a 24 h DTH reaction in tempo as well as by an absence of induration and paucity of cellular infiltrate as revealed by biopsy. This activity in the MLC derived supernatant is likely to be unrelated to that facilitating the DTH reaction as supernatants raised with PPD did not elicit this early response.

The identity of the mediator or mediators participating in this response, its mode of action, and its role in a normal DTH reaction are not yet known, and are the subject of current experiments. As to its identity we ruled out it being IL-2 by recovering activity in supernatants which had been depleted of IL-2 on an immunosorbent column constructed with anti-IL-2 antibodies. Regarding its mechanism of action we entertain the following working hypothesis. In DTH the antigen injected must interact with antigen-sensitive cells for a reaction to occur, which in turn is detected as an accumulation of mononuclear cells (Askanase & Van Loveren, 1983; Waksman, 1979). This sequence does not take place in anergic patients without coinjection of the mediator. One interpretation for the role of the mediator is that its function is initially to attract cells to the injection site where interaction between these cells and antigen results in the release of secondary mediators attracting a second wave of cells characteristic of the DTH reaction. According to

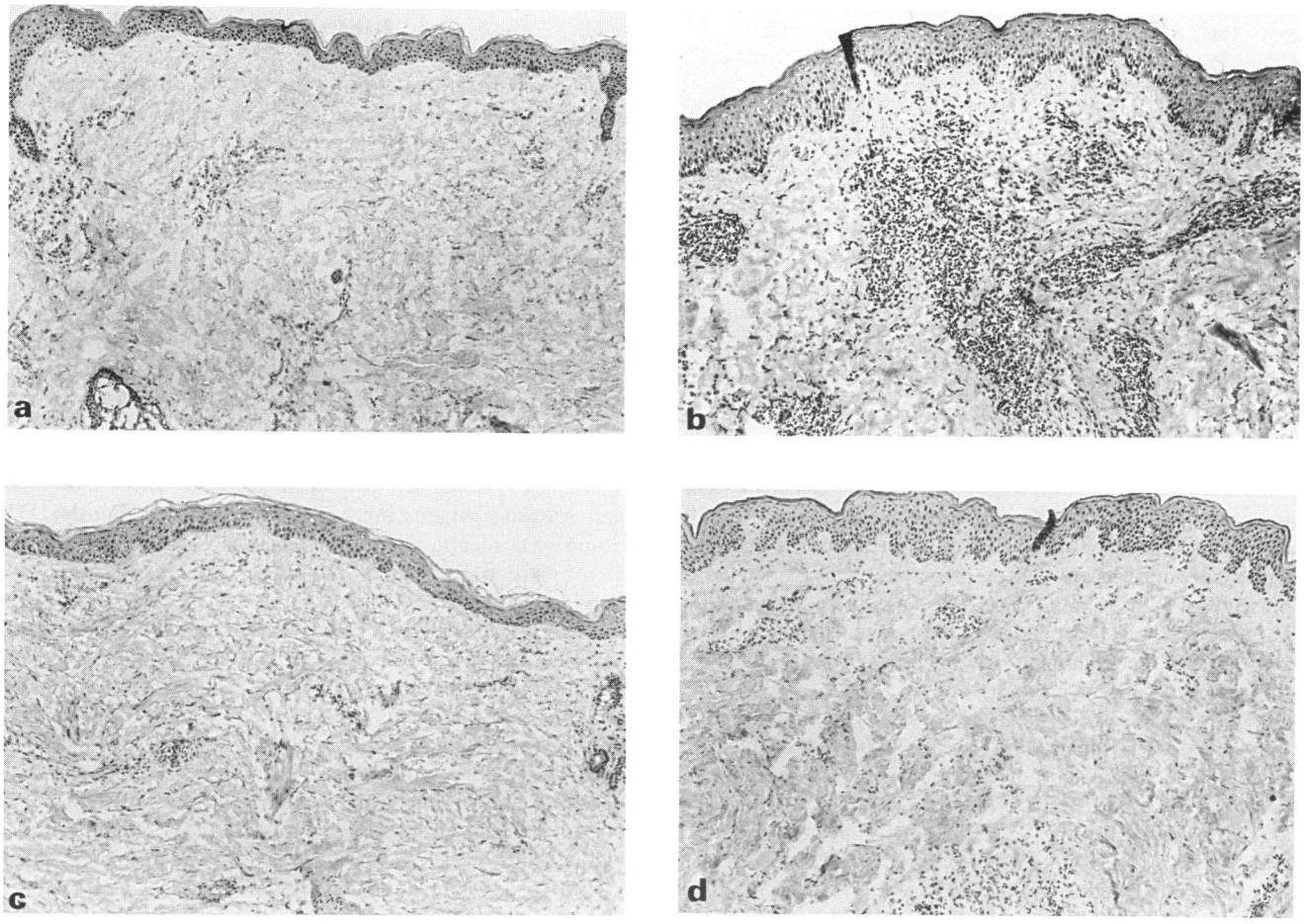

Fig. 1. Skin biopsies obtained from anergic patients injected (a) with PPD alone, or (b) with PPD and MLC supernatant 24 h after skin testing. Figures 1c and 1d are biopsies of 12 and 24 h skin test sites from normal individuals injected with MLC supernatant 12 and 24 h earlier, respectively. The only positive reaction is that shown in (b) (haematoxylin, × 200).

this interpretation, the defect in anergy lies in chemotaxis. The fact that only very few cells can be detected at the site of injection of the mediator without antigen is not incompatible with this view. An alternative explanation would ascribe another role to the mediator, i.e. to deliver a second, activating signal, along with antigen to initiate the DTH reaction cascade. This function could by itself reconstitute the response, or it could be a second activity present in the supernatant associated with the same or with a different molecule.

What we do know at the moment is that the DTH reaction is defective in anergic individuals because a component, probably early, of the reaction which is mediator driven is deficient, and that replacement of the mediator allows the reaction to occur. An understanding of how the mediator functions will explain the defect in anergy, which in turn will define a component in the cascade of events in the normal DTH response.

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