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Deficiency of Interleukin-2 Production and Interleukin-2 Receptor Expression on Peripheral Blood Leukocytes after Phytohemagglutinin Stimulation in Pemphigus

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Peripheral blood mononuclear cells from 22 pemphigus patients with active disease and 30 normal subjects were evaluated for interleukin 2 (IL-2) production and IL-2 receptor expression following stimulation with phytohemagglutinin P (PHA-P). The IL-2 levels were lower in patients compared to corresponding controls and the production was delayed after PHA stimulation. This deficiency was most pronounced in severely affected patients. IL-2 receptor appearance also was lower after PHA stimulation in a small number of patients tested. These results indicate that some cellular immune functions are altered in pemphigus.

Pemphigus is an autoimmune, often fatal disease involving the skin and mucous membranes. There is in vivo deposition of IgG in the intercellular cement substance of the epidermis [1]. The serum contains an anti-intercellular cement substance (ICS) antibody of the IgG class [2]. The titer of the antibody correlates with disease activity and severity [3]. When passively transferred into neonatal BALB/c mice, the antibody caused clinical and immunopathologic disease [4]. A decrease in the total number of T cells and a T-cell depletion in the paracortical areas of the lymph nodes have been observed in some pemphigus foliaceus patients [5].

A study was undertaken to investigate the possible changes in the critical lymphokine, interleukin 2 (IL-2) and its receptor in pemphigus. IL-2 biosynthesis and IL-2 receptor expression were studied in patients with active pemphigus and correlated with disease activity, severity, and anti-ICS antibody titer. In further studies proliferative response to phytohemagglutinin P (PHA-P) was measured in patients and compared to normals and the effects of purified IL-2 on the proliferative response was explored.

MATERIALS AND METHODS

Patients and Controls

Twenty-two pemphigus vulgaris patients ranging in age from 18–82 (mean 58 ± 18) years, seen at the UCLA Medical Center were studied. The patients had acute disease at the time of the initial study and were not on any systemic therapy. The diagnosis was made on the basis of clinical features, routine histology, and immunofluorescent studies [6]. Patients were divided into categories of severity on the basis of clinical involvement and anti-ICS antibody titers. Patients with mild or 1+ disease had less than 30 lesions usually in one anatomic location and had anti-ICS antibody titers of 0–40. Patients with moderate or 2+ disease had 40–80 lesions in different anatomic areas and anti-ICS antibody titers of 80–320. Patients with 3+ or severe disease had over 80 lesions in generalized distribution and antibody titers of 640 or greater. Serum from each individual was assessed from anti-ICS antibody of the IgG isotype by the indirect immunofluorescence technique using monkey esophagus as the substrate [7]. Thirty normal individuals ranging in age from 20–75 (mean 38 ± 14) years and 5 patients with other dermatologic diseases served as controls. The patient and control groups had similar sex distributions.

Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized (20 units/ml) blood by the Ficoll-Hypaque gradient method [8]. Harvested cells were washed with RPMI 1640 (Irvine Scientific, Santa Ana, California) 3 times. Cells with greater than 95% viability were adjusted to 1×10^6/ml with RPMI 1640 containing penicillin (25 units/ml), streptomycin (25 ng/ml), glutamine (2 mM) (Irvine Scientific), and 0.25% bovine serum albumin (BSA) (Sigma, St. Louis, Missouri). Wright's stain examination of the suspensions demonstrated the cell population consisted of 10–20% monocytes and 1–10% polymorphonuclear leukocytes.

Interleukin-2 Production

PBMC (1×10^6) from patients or controls were cultured in 0.25% BSA with 10 μg phytohemagglutinin P (PHA-P) (Difco Laboratories, Detroit, Michigan) for 24, 48, 72, 96, and 120 h. To determine the capacity of each specimen to produce IL-2, at various times the cells were removed by centrifugation and the supernatants were individually collected and tested on a mouse cytotoxic cell line (CTLL-2) for growth-promoting activity [9].

Biologic Assay for IL-2 Activity

Individual samples were tested for IL-2 activity by their ability to stimulate CTLL-2 to incorporate tritiated thymidine [9]. The assessment of the IL-2 activity in units/ml was based on linear interpolation analysis of standard to normal and calculated according to the following formula [10].

$$\text{IL-2 activity of sample (units/ml)} = \frac{\text{Reciprocal titer of test sample at 30\% of maximal cpm of standard}}{\text{Reciprocal titer at 30\% of maximal cpm of standard}}$$

where 100 is the number of units/ml for standard.

Responsiveness to PHA-P

The response of PBMC to PHA-P was studied. PBMC (2×10^5) from patients or controls were cultured for 72 h in a Cook microtiter plate (Microbiological Associates, Los Angeles, California) with 10 μg PHA-P/ml and 10% fetal calf serum (FCS) (Irvine Scientific) as described earlier [11].

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Abbreviations:
BSA: bovine serum albumin
CTLL-2: mouse cytotoxic cell line
FCS: fetal calf serum
ICS: intercellular cement substance
IL-1: interleukin 1
IL-2: interleukin 2
PBMC: peripheral blood mononuclear cells
PHA-P: phytohemagglutinin P
TPA: 12-*O*-tetradecanoylphorbol-13-acetate

Proliferative Effect of IL-2

The enhancing effect of IL-2 on PBMC response to PHA-P was determined. In preliminary experiments IL-2 ranging from 1–30 units per 1×10^6 cells in cultures was added. The cultures contained 2×10^5 cells. IL-2 was added 10 min to 1 h after PHA stimulation. IL-2 was prepared and purified as previously described [12].

Enumeration of IL-2 Receptor-Bearing Cells

Quantification of IL-2 receptor-bearing cells was performed on fresh or cultured mononuclear cells. Tac antigen expressed on surface of lymphocytes was determined using a flow cytometer (Spectrum III, Ortho Diagnostics System, Westwood, Massachusetts). The PBMC cells, fresh and cultured, were incubated for 45 min with murine monoclonal anti-Tac antibody (provided by Dr. Waldman, NIH, Bethesda, Maryland, washed, incubated for an additional 45 min with fluorescein-conjugated rabbit antimouse Ig antisera (Becton-Dickinson, Mountain View, California), and enumerated by the automated flow cytometer. Murine IgG_{2a} myeloma protein (RPC-5, Bionetics, Inc, Bethesda, Maryland) was used as control to determine nonspecific binding.

Statistics

Two group comparisons were done using the t-test and the Wilcoxon rank sum test. A significant result was reported if it was significant on both tests. As the comparisons were done at 5 prespecified time points, the p value was adjusted for multiple comparisons using Bonferroni's method [13].

RESULTS

IL-2 levels were determined at 24, 48, 72, 96, and 120 h in each patient and control following mitogenic (PHA) stimulation (Fig 1). At 24 and 48 h, the level of IL-2 production was significantly lower in patients when compared to controls ($p < 0.05$). In the control group, IL-2 production rapidly increased and attained maximum levels after 48–72 h and declined after 96–120 h. In the pemphigus patients, IL-2 production was generally delayed and its level did not increase as rapidly as in the control group.

Patients with mild or moderate disease produced decreased levels of IL-2 at 24 and 48 h when compared to controls (Fig 2). The differences were not, however, statistically significant. IL-2 levels gradually increased in most patients with mild or moderate disease after 24 h. Those with severe disease showed the lowest IL-2 levels at 24, 48, and 72 h. When compared to controls, this group showed significantly reduced IL-2 production at 24 and 48 h ($p < 0.05$). At 96 and 120 h readings were available for less than one-half and one-third, respectively, of patients and controls. Thus results obtained at these times should be cautiously interpreted due to the small number of patients studied at these time intervals.

Repeat tests were performed on 4 patients with severe disease and 4 controls to determine reproducibility. Data shown in Table I confirm that IL-2 production in these 4 patients with severe disease was similarly depressed on repeat observations.

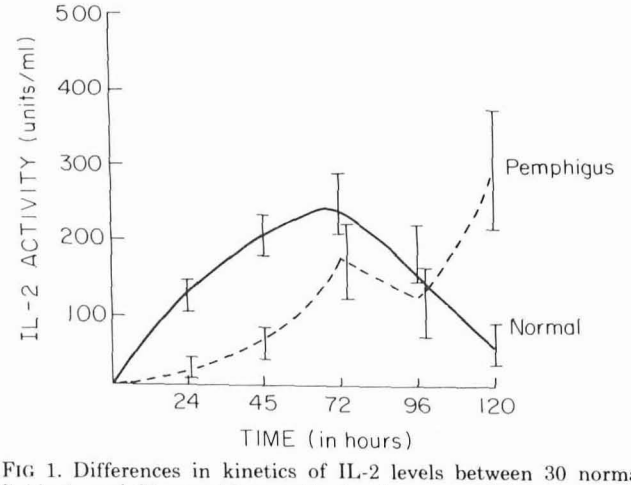

FIG 1. Differences in kinetics of IL-2 levels between 30 normal individuals and 22 pemphigus patients who were mildly to severely affected.

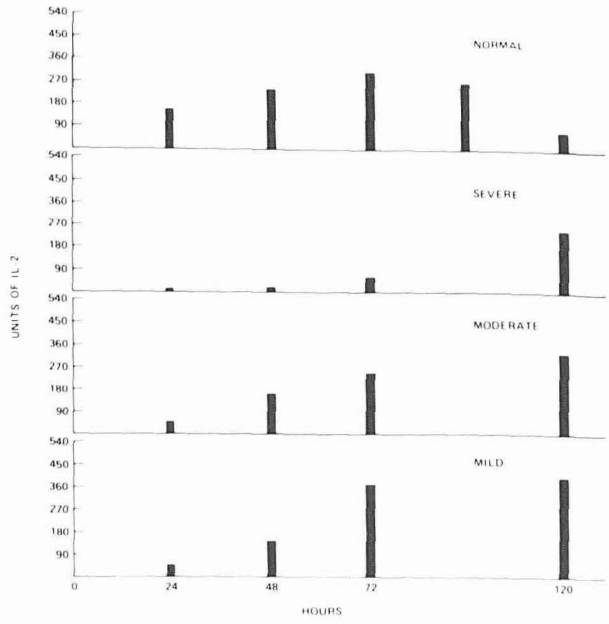

FIG 2. Comparison of IL-2 biosynthesis in pemphigus patients (11 severe, 6 moderate, and 5 mildly affected). Thirty normal individuals were tested simultaneously.

IL-2 is known to play a major role in proliferative response to mitogens, such as PHA. The PBMC from the 10 patients and the 10 controls demonstrated a wide range of proliferative responsiveness with mean [^{3}H]thymidine uptake of $120 \pm 60 \times 10^3$ cpm for normals and $60 \pm 36 \times 10^3$ for patients (Fig 3). However, if patients with severe disease are segregated there is a marked reduction in the PHA response. These patients showed [^{3}H]thymidine incorporation of only $27 \pm 5 \times 10^3$ cpm compared to $120 \pm 60 \times 10^3$ cpm for controls. The difference between these values is not statistically significant. However, it is possible that this is a real difference but that the small sample size does not allow this to be demonstrated statistically.

IL-2 receptor expression after PHA stimulation was evaluated in several experiments. Tac antigen (IL-2 receptor) expression was gradually increased on cultured PBMC from 0 to 72 h in both 7 normals and 7 patients (Fig 3). The frequency of expression of the IL-2 receptor was generally less in patients compared to the controls at each time interval. This difference was more pronounced in patients with severe disease compared to those with mild or moderate activity (data not shown).

Preliminary studies indicated that those with high response to PHA often had little response to added IL-2. Therefore PBMC from 5 normals and 5 patients with low PHA response (below 50×10^3 cpm) were exposed to PHA and purified IL-2 (10 units/10^6 cells) and then the proliferative response was assessed after 72 h of culture. The cultures containing IL-2 in addition to PHA showed a greater magnitude of [^{3}H]thymidine incorporation in patients and controls than cultures containing PHA or IL-2 alone (Fig 4). Although the statistical significance of this result is not determined due to the small sample size, the increment in the proliferative response was greater than the sum of the responses to PHA and IL-2 separately, indicating that IL-2 had some enhancing effect on proliferation in these cultures.

DISCUSSION

IL-2 is a soluble factor produced by stimulated T cells [14]. Both helper/inducer and suppressor/cytotoxic T cells interact

TABLE I. *Kinetics of IL-2 production observed in repeat testing*

Donor no.	Age/Sex	Severity of disease	Antibody titer	IL-2 activity (units/ml) from PBMC at:				
				24 h	48 h	72 h	96 h	120 h
				Pemphigus				
12A[a]	53 F	3+	640	0	17	15	12	13
12B	53 F	3+	640	0	0	0	0	0
10A	70 M	3+	320	24	N.D.[b]	N.D.	N.D.	N.D.
10B	71 M	3+	320	13	27	11	N.D.	N.D.
11A	39 F	2+	160	0	0	0	0	0
11B	39 F	3+	320	0	0	0	15	4
8A	70 F	3+	320	2	4	3	3	3
8B	70 F	3+	320	0	0	0	0	0
				Normal				
24A	43 M	—	—	270	350	250	20	20
24B	43 M	—	—	N.D.	182	62	N.D.	N.D.
22A	75 M	—	—	32	138	N.D.	N.D.	N.D.
22B	75 M	—	—	33	227	750	750	N.D.
23A	22 M	—	—	234	170	N.D.	N.D.	N.D.
23B	22 M	—	—	N.D.	138	N.D.	N.D.	N.D.
20A	70 F	—	—	109	221	97	32	12
20B	70 F	—	—	68	98	9	N.D.	N.D.

[a] A and B represent repeat of samples from same individual at two different intervals.
[b] N.D. = not done.

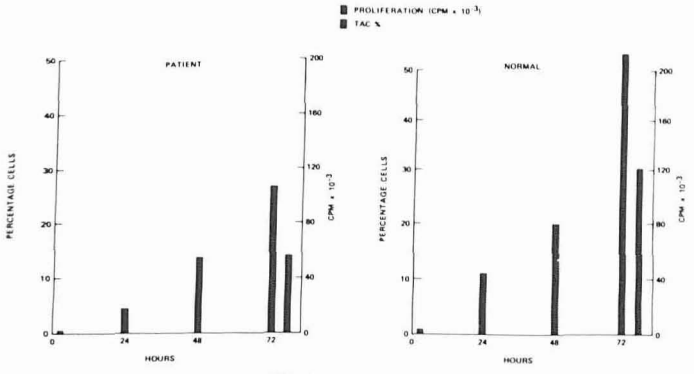

FIG 3. Kinetics of Tac (IL-2 receptor) expression in 10 pemphigus patients (mildly to severely affected) and 10 normal individuals. PHA proliferative response of these patients and normals at 72 h. Cultures are included.

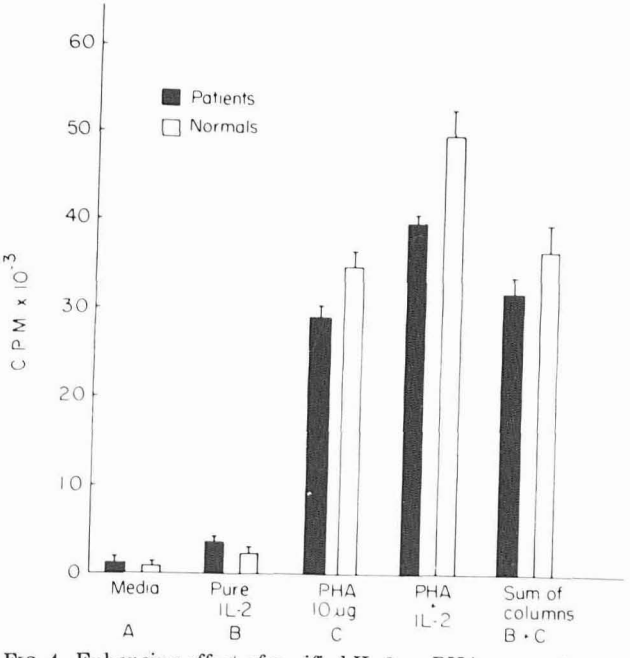

FIG 4. Enhancing effect of purified IL-2 on PHA responsiveness of peripheral blood mononuclear purified IL-2 on cells from 5 normal individuals and 5 pemphigus patients (severe). Individuals selected in this experiment exhibited PHA responses of less than 50×10^3 cpm. Each *bar* represents SEM of each test.

with IL-2 molecules via specific receptors [15,16]. Such receptors are detected by the anti-Tac monoclonal antibody [15]. Both IL-2 production and IL-2 receptor expression are key events in the cascade of cellular events leading to proliferation of increased T cells and preparation for immune function [10,17,18]. In several strains of mice that develop autoimmune disease, deficiencies of IL-2 production have been reported [19,20] that correlate with disease manifestations and severity. Recent observations indicate that such phenomena also may be present in human patients with systemic lupus erythematosus [21,22].

In this study PBMC of patients with pemphigus responded to PHA with lower levels of IL-2 activity in culture supernatants compared to normals. This decreased activity is more pronounced in patients with severe disease and higher anti-ICS antibody titers. Decreased IL-2 receptor expression also paralleled the disease extent and anti-ICS antibody titers.

Several studies have focused on the antibody aspects and the autoimmune nature of pemphigus. This study emphasizes the coexistence of disorders of cellular immune regulation in pemphigus. While it is not clear whether these cellular immmune changes are primary or secondary to the disease, it is possible that they contribute to some of the manifestations. Indeed, disorders of immune regulation are postulated to be the basis for expression of autoantibodies [23].

The T-cell changes in pemphigus described here are the slow generation of IL-2 and reduced expression of IL-2 receptor. These could result from excessive suppressor activity, either of monocytes or of T cells. Preliminary efforts, however, to demonstrate excessive suppressor activity did not support this possibility. In 4 experiments, PBMC from pemphigus patients were irradiated with 1000 rad or passed through nylon wool columns to reduce suppressor cell activity [24] or were treated with indomethacin, a known inhibitor of prostaglandin E; there was no change in IL-2 production. No evidence of suppression of IgG secretion was observed in one study [25]. In another study no loss of suppressor T-cell function was observed in mononuclear cells from pemphigus patients using concanavalin A induction techniques [26].

Deficiency of IL-1 production or response to IL-1 can result in the decreased production of IL-2. In additional studies when 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an agent that at least in some systems mimics the activity of IL-1 [16,27], was added to cells from 4 patients' cells that had negligible IL-2

production, and no significant alteration of IL-2 production was observed. However, the role of exogenous IL-1 instead of TPA was not tested.

In additional preliminary studies, it was observed that exogenous IL-2 can exert an enhancing influence on cellular response to PHA. This effect was pronounced in individuals with a decreased proliferative response. However, the patients with low PHA response were compared to normals with low PHA response also. This was done to eliminate the variable of baseline response and, hence, it appears that the enhanced effect of exogenous IL-2 is a demonstration of the effect of IL-2 on patient PBMC. The significance of this result remains to be elucidated.

Further studies are needed to enhance the understanding of the cellular and molecular mechanisms of autoantibody formation and the role of cells of the immune system in the pathogenesis of pemphigus.

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