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T- and B-Lymphocytes in Pemphigus vulgaris

A Clinico-Immunological Follow-up Study of Fifty Patients with Pemphigus vulgaris

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Peripheral blood T-lymphocytes, studied in 50 patients with pemphigus vulgaris by means of spontaneous (E) and active (EA) rosette-formation, were increased in active disease and gradually returned to normal after achieving clinical remission of pemphigus vulgaris. The portion of B-cells quantified by M-rosette formation was increased in active disease as well (19.9±3.0% vs. 10.8±1.6% in controls) ($p<0.02$) but returned to normal immediately after clinical remission was obtained. Levamisole in vitro suppressed E-, EA-, and M-rosette formation in all the patients examined with active disease. The subsets of T-cells in 10 non-treated patients with active pemphigus vulgaris and 5 patients with a long-term steroid maintained remission of the disease were studied using OKT series monoclonal antihuman antibodies. A statistically significant increase in the percentage of OKT3+ cells ($p<0.05$) and a considerable increase in OKT4+ cell count ($p<0.002$) were registered in patients with active pemphigus vulgaris, compared with controls. In five patients with 2–9 years' remission the percentage of OKT3+, OKT4+, OKT5+ and OKT8+ cells did not significantly differ from controls. *Key words: Rosette-formation assays; Monoclonal antibodies; Lymphocyte blast transformation; Levamisole.* (Received May 30, 1986.)

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In the last decade several reports appeared evaluating the role of cellular immunity disturbances in the pathogenesis of pemphigus vulgaris (PV) as well as in the other forms of pemphigus. The reported results are, however, controversial. A decreased level of E-rosette forming cells (E-RFC) and normal contents of Fc receptor bearing cells or B-cells, and a normal PHA response were revealed in active PV (1, 2), and it was suggested that the decrease in T-cell count could be due to a true lack of these cells or to their relative immaturity (2). The decrease in the total T-lymphocyte count (E-RFC) and in the number of T-cells forming active E-rosettes (functional T-lymphocytes) as well as the normal level of EAC-rosette forming cells were detected in patients with pemphigus foliaceus (3). The reduced T-cell count in blood and a weak PHA stimulation of lymphocytes in pemphigus vegetans have also been reported (4). A massive increase of the OKT4 reactive helper T-cell population, and a decrease in OKT5 and OKT8 reactive suppressor cells in PV was demonstrated (5). In this last study it was suggested that the expansion of the helper T-cell population and the inability of suppressor T-cell population to mediate feedback suppression lead to unrestrained antibody production, with some of these antibodies being autoreactive and antiepidermal in nature. A significantly low activity of suppressor T-cells in vitro has also been demonstrated using the Con-A suppressor assay (6), whereas in another study an unimpaired suppressor T-cell activity was shown (7). A study of T-cell subset distribution in PV by means of Leu-1, -2, and -3 monoclonal antibodies failed to demonstrate any significant alterations in the relative percentages of total T-cells (Leu-1 reactive) or T-cells expressing T-helper (Leu-3) or T-suppressor (Leu-2) phenotype in the

peripheral blood of patients with active disease compared to those with inactive disease or healthy controls (8). The circulating B-cells in patients with PV have been reported to be increased (9).

In the present study different assays were used to evaluate the state of cell-mediated immune system and the distribution of T-cell subpopulations in the peripheral blood of patients with different severity of PV.

MATERIAL AND METHODS

Patients

Fifty patients with PV, 17 males and 33 females, 31–81 years of age (the mean 56 years) were included in the study which was performed in 1977–1984 in the Department of Dermatology of Moscow Medical Stomatological Institute. The diagnosis of PV in all of the patients was confirmed clinically, cytologically (acantholytic cells in smears), histologically and, except of four cases, by means of direct and indirect immunofluorescence. In 37 patients the disease started from lesions on oral mucous membrane, and a further 13 patients had initial lesions on the skin. In 27 patients the study was performed before treatment, 13 patients were examined in the period of the relapse of PV which happened despite steroid administration. Five patients had 2–9 years' remission maintained by a low daily dose of steroids. Nine more patients who went into remission during the time of our study did not have any relapses of the disease within 1–2 years' follow-up. Five other patients with proven PV in the past at the time of the last follow-up visit had complete remission of the disease from 7 to 21 years, being without treatment for 3–12 years. Seventeen patients had pemphigus lesions only on the oral or laryngeal mucous membrane, and eight only on the skin. Fifteen patients had both skin and mucous membrane lesions. The general state of 12 patients was estimated as severe.

Thirty healthy volunteers, 11 males and 19 females, 25–76 years of age (the mean 52.5 years) served as controls.

Lymphocyte isolation

Peripheral blood mononuclear cells were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. After washing the cells three times with Hanks' balanced salt solution (HBSS), unfractioned cells were centrifuged at 200 g for 10 min. Residual red blood cells were lysed by 0.2% aqueous sodium chloride with further isotonicity restoration by 1.6% sodium chloride. Cells were rinsed again with HBSS, centrifuged and resuspended at 5×10^6 cells/ml in Medium 199 (Gibco). Cell suspension consisted of 70–80% lymphocytes, 20–30% monocytes, and 1–15% polymorphonuclear leukocytes, as determined by Wright's stain. The cell viability was consistently greater than 95% by the criterion of trypan blue exclusion.

Membrane marker studies

The techniques for spontaneous and active T-rosettes (E-RFC and EA-RFC, respectively) have been previously described. Briefly, for detecting the E-RFC (10), 0.1 ml of human lymphocyte suspension (HLS) was mixed with 0.1 ml of 0.5% sheep red blood cells (SRBC) and incubated at 37°C for 5 min. The mixed cell suspension was spun at 200 g for 5 min and incubated for 1 h at 4°C. The supernatant was removed and the top layer of the pellet was gently resuspended by shaking. The percentage of E-RFC was determined in a hemocytometer. Three hundred cells were counted, and those which bound three and more SRBC were considered rosettes.

For EA-RFC detection (11), 0.1 ml of HLS (5×10^6 cells/ml) was incubated for 1 h at 37°C with 0.1 ml of inactivated fetal calf serum (FCS), then, SRBC in saline were added to obtain a final ratio of eight SRBC to one lymphocyte. The tubes were centrifuged for 5 min at 200 g and resuspended. Cells binding at least three SRBC were considered rosettes. Three hundred cells were counted using a hemocytometer.

A subset of B-cells was quantified by the M-rosette assay with mouse red blood cells (MRBC) as described by Forbes & Zalewski (12). MRBC, obtained from the ophthalmic venous plexus of mice and collected into saline containing citrate (3.8%) were washed three times in 0.9% saline, then 0.1 ml of packed cells were resuspended in 10 ml of Medium 199. 0.25 ml of MRBC suspension supplemented with 0.1 ml of FCS and 0.025 ml of HLS were incubated for 5 min at 37°C, centrifuged at 200 g for 5 min and incubated at 4°C for 30 min. 0.025 ml of 2.5% glutaraldehyde in Medium 199 was added, the cells resuspended gently, and left at room temperature (22°C) for 15 min. The cells were stained with 0.5% toluidine blue. Lymphocytes which bound two or more MRBC were considered to be rosettes (M-RFC).

The effect of levamisole on the rosette-forming capabilities of lymphocytes in vitro was evaluated as follow (13): 0.1 ml of HLS (5×10^6 cells/ml) was preliminarily incubated with 0.15 ml of levamisole (10 µg/ml) for 1 h at 37°C, washed twice in HBSS, and tested for E-, EA-, and M-rosettes.

Monoclonal antibodies OKT3, OKT4, OKT5 and OKT8 (Ortho Pharmaceutical, Raritan, N.J., USA) were used for the detection of T-cell subsets. Lymphocytes isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation were three times washed with HBSS, suspended with 20% FCS in RPMI-1640 (Gibco) and incubated at 37°C overnight in Petri dishes to deplete monocytes. Nonadherent cells were collected by rinsing the plates with HBSS, centrifuged, resuspended in RPMI-1640 supplemented with 5% FCS and 1% sodium azide at 5×10^6 cells per milliliter and stored in liquid nitrogen until testing. The obtained cell suspension (0.2 ml) was incubated for 45 min with 10 µl of reconstituted monoclonal antibody solution at 4°C, twice washed and resuspended in 0.1 ml wash medium. Then the cells were incubated with 0.1 ml of diluted (1 : 10) fluorescein-conjugated goat antimouse IgG (G/M FITC, Nordic Immunological Laboratory, Tilburg, The Netherlands) for 30 min, washed three times and resuspended in wash medium supplemented with 3 mM EDTA. Fluorescein-labeled cells were counted using a Leitz Orthoplan microscope.

Lymphocyte transformation

In the test of lymphocyte blast transformation phytohaemagglutinin (PHA M, Difco), in concentration 250 µg/ml, was used as mitogen. 0.2 ml of HLS (1×10^6 cells/ml) supplemented with 0.05 ml PHA was incubated at 37°C for 72 h, centrifuged at 200 g for 10 min, and sediment separated. One thousand lymphocytes were counted, and the percentage of cells transformed into blast-like forms was detected. The same lymphocyte suspension (1×10^6 cells/ml) without mitogen served as control.

Statistical analysis

All the numerical results were statistically analysed by Student's t-test. As a level of significance $p<0.05$ was adapted.

RESULTS

An absolute (1 370±88 cells/mm^3 vs. 910±20 cells/mm^3 in healthy controls) and relative (72.2±2.7% vs. 61.2±0.8%) increase of T-lymphocytes in the peripheral blood was revealed in 40 patients with active PV ($p<0.01$). The percentage of active T-lymphocytes in these patients was increased as well (44.3±3.2% vs. 29.4±4.1% in controls) ($p<0.02$). At the same time incubation with levamisole induced a decrease in the E-RFC and EA-RFC counts in all of these patients (Table I). The study of M-RFC also demonstrated their absolute (595±54 cells/mm^3 vs. 300±10 cells/mm^3) and relative (19.9±3.0% vs. 10.8±1.6% in controls) increase in all of these patients ($p<0.02$). The decrease of M-RFC occurred in the test with levamisole (Table I).

The study of lymphocytes in 30 patients within 7–10 days after clinical remission was achieved under treatment (no new lesions, healing of existing erosions) showed that levamisole in vitro began to stimulate EA-rosette formation whereas its effect on E-rosette formation remained suppressive.

In 14 patients with steroid maintained remission lasting 1–9 years the parameters studied were gradually returning to normal but the levamisole-induced suppression of E-RFC was still detectable (not statistically significant data, $p>0.05$).

Because of the continuous follow-up of patients, five of them happened to be examined just before a relapse of the disease, which occurred despite the maintenance steroid treatment. The increase in the E-RFC and EA-RFC counts which again took place in these patients exceeded that which was registered before the beginning of steroid treatment.

Five patients with 7–21 years of PV remission and without treatment for 3–12 years, had normal indices of E-, EA- and M-RFC.

The results of the detection of T-cell subset distribution in the peripheral blood of 15 patients with PV and 15 healthy controls are presented in Table II. A slight but statistically significant increase of OKT3 reactive cells was found in 10 patients with active disease compared with controls ($p<0.05$). A considerable expansion of OKT4+ cells ($p<0.002$)

and a depletion of OKT5+ and OKT8+ cells were revealed in all of these 10 patients with active disease compared with controls. The percentage of T-cell subsets (OKT3+, OKT4+, OKT5+ and OKT8+) in peripheral blood of five patients whose long-term remission (2–9 years) was maintained by 5–10 mg of prednisolone a day, did not significantly differ from controls.

Lymphocyte transformation with PHA performed in 25 patients with active PV revealed a weak response to mitogen stimulation (Table III). The mean percentage of lymphocytes

Table I. *Mean percentages of E-rosette forming cells (E-RFC), EA-rosette forming cells (EA-RFC), and M-rosette forming cells (M-RFC) in the peripheral blood of patients with different severity of pemphigus vulgaris (PV) and the effect of levamisole in vitro on these parameters (%mean±SD)*

Patients	E-RFC	E-RFC with levamisole	EA-RFC	EA-RFC with levamisole	M-RFC	M-RFC with levamisole
Active PV, $n=40$	72.2±2.7[a]	55.8±2.0[a]	44.3±3.2[b]	35.5±2.3	19.9±3.0[b]	14.7±2.1
7–10 days after achieving clinical remission, $n=30$	69.3±1.1	66.2±1.4	39.0±2.9	49.3±2.5	12.2±2.7	13.4±2.2
Long-term prednisolone maintained remission						
(*a*) 10 mg/day, $n=8$	64.7±3.4	59.1±2.1[NS]	34.7±4.4	41.3±2.7	ND	ND
(*b*) 5 mg/day, $n=6$	62.4±2.2	60.3±4.9[NS]	31.3±3.5	40.4±3.6	ND	ND
Period preceeding exacerbation, $n=5$	73.0±2.1[b]	58.8±2.9	50.9±3.3	43.6±2.4	ND	ND
Remission without maintenance treatment, $n=5$	63.2±2.3	71.7±3.2	31.2±3.7	38.8±5.4	11.9±1.1	15.3±4.1
Healthy controls, $n=30$	61.2±0.8	63.5±5.2	29.4±4.1	38.5±5.0	10.8±1.6	16.4±2.4

[a] $p<0.01$. [b] $p<0.02$.
p values indicate differences compared with healthy controls. NS=not statistically significant ($p>0.05$). ND=not done.

Table II. *The distribution of T-lymphocyte subsets in the peripheral blood of patients with pemphigus vulgaris (PV)*

Patients	Cell counts (%mean±SD)				
	OKT3	OKT4	OKT5	OKT8	OKT4/OKT8
Active PV, $n=10$	78.4±2.3 $p<0.05$	65.9±5.3 $p<0.002$	9.8±3.9 $p<0.01$	12.2±4.5 $p<0.02$	5.49 $p<0.01$
Long-term steroid maintained remission, $n=5$	74.0±6.1	49.1±4.8	21.5±3.0	24.7±4.2	1.99
Controls (sex- and age-matched), $n=15$	73.2±3.7	48.5±5.4	22.1±5.9	25.6±4.6	1.89

p values indicate differences between active PV and controls.

transformed into blast-like forms in the presence of PHA was only 14.4±1.8% ($p<0.005$). In the control study without mitogen the quantity of spontaneously transformed lymphocytes was 6.1±1.0% (compared with 1.2±0.3% in healthy controls).

DISCUSSION

The disturbance of T-cells was most pronounced in the active period of the disease, especially in patients with severe PV. Thus a pronounced imbalance in the OKT4/OKT8 ratio was revealed in these patients, which was a result of the increase of OKT4+ cell population and a depletion of OKT5+, OKT8+ cell population. These data are in line with those previously reported (5, 6) but in conflict with others (8), where no alterations in the cell mediated immune system were found in PV patients compared with controls. Monoclonal T4 and T8 antibodies recognize differentiation markers characteristic for helper/inducer and suppressor/cytotoxic T-lymphocyte subsets, respectively. The low PHA response of peripheral blood mononuclear cells in PV patients therefore, considering the high T4/T8 ratio, seems surprising. However, the situation in this respect could be somewhat similar to that in other diseases, where T-lymphocytes are preactivated already under in vivo conditions and show a diminished PHA response in vitro (14). Furthermore, there is often no direct correlation, for example, between T8 expression and suppressor/cytotoxic function. For this reason one should be careful when judging the in vivo role of OKT8+ cells.

The changes in T-lymphocytes diminished with the disappearance of clinical manifestations of the disease under treatment. Complete restoration of the T-cell indices occurred only in patients with long-term remission of the disease maintained for years by minimal daily dose of corticosteroids. The quantity of M-RFC was normal immediately following steroid-induced clinical remission. M-rosette assay delineates a portion of B-cells which, according to Forbes & Zalewski (12), possess surface membrane immunoglobulins of all classes. The proportion of different classes of immunoglobulin on the surface of M-RFC does not differ from that in the total B-cell population. Even if it is not the most reliable marker for B-lymphocytes, the obtained data are in keeping with the suggestion that corticosteroid administration in PV results in the suppression of B-cells (15). It has also been reported that corticosteroids have differential effects on the lymphocyte populations involved in immunoglobulin biosynthesis: B-cell responsiveness is diminished, suppressor

Table III. *The results of PHA induced and spontaneous lymphocyte transformation in 25 patients with active pemphigus vulgaris (PV) and 30 sex- and age-matched controls (%mean±SD)*

Examined group	Stimulation	Blast forms	Unchanged lymphocytes
Active PV, $n=25$	PHA	14.4±1.8 $p<0.005$	85.9±1.6
	Without mitogen	6.1±1.0 $p<0.02$	93.9±1.2
Healthy controls, $n=30$	PHA	78.0±2.0	21.1±1.5
	Without mitogen	1.2±0.3	98.8±0.4

p values indicate differences compared with controls.

T-lymphocyte activity is removed, and helper T-lymphocyte function is unaffected (16). If the patients with PV have a decreased suppressor cell activity, which is possibly suggested by low percentage of T8 cells, the main effect of corticosteroids could be due to inhibition of B-cell activity and the ability of B-cells to respond to helper T-lymphocytes.

Levamisole previously considered only as an immunostimulant is now regarded as immunomodulator which affects the surface markers of all immune cells (17, 18). The augmentation in the lymphocyte response under levamisole action is not always present, and detectable only for a narrow concentration range of the drug (13). Wybran & Govaerts (13) who studied the action of levamisole in vitro on E-, EA-, and EAC-rosette formation in healthy subjects, suggested that it increases the expression of T-cell receptor and decreases that of C3 receptor. The present results confirm that levamisole indeed affects lymphocyte surface markers and, although the exact mechanisms responsible for the therapeutic effect of the drug are not enough clear, these interactions between levamisole and lymphocyte surface structures may form the basis for its immunomodulatory action. The suppressive effect of levamisole on lymphocyte rosette formation in vitro, which was revealed in our patients, probably might be due to the very special immune changes occurring in PV. The change of levamisole action in vitro from suppression to stimulation in the test of E-rosette formation was registered in patients with long-term remission of PV parallel with the disappearance of the circulating pemphigus antibodies (19).

The autoimmune mechanisms of PV pathogenesis, and the primary role of autoantibodies in this disease have been reported by many investigators. The documented alterations in the cell mediated immune system which controls and regulates antibody production, provide further support for the autoimmune nature of PV.

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