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Increased Chemiluminescence of Whole Blood and Normal T-lymphocyte Subsets in Severe Nodular Acne and Acne Fulminans

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To investigate the inflammatory and immunological aspects of severe acne, we examined the luminol-enhanced chemiluminescence of whole blood, T-cell subsets and natural killer cell functions in 11 patients with severe nodular acne and 4 patients with acne fulminans. In patients with severe nodular acne, the active phase of the disease, compared to the values in remission (means 47 mV, SD 24.8 and 32 mV, SD 8.3, $p < 0.05$). The patients with acne fulminans also showed high values in the active phase of the disease (mean mV 68.3, SD 3.5) compared to remission (mean 30.5 mV, SD 15.3). No marked alterations were seen in the percentages of T-helper cells, T-suppressor cells or DR-positive lymphocytes or in the levels of soluble interleukin 2 receptor. The percentages and activities of natural killer cells did not show any significant changes either. Five patients (4 with severe nodular acne and one with acne fulminans, accounting for 33% of all patients) carried HLA Cw6 antigen, which is a significantly increased frequency compared to healthy controls ($pc = 0.015$). The present chemiluminescence results suggest that peripheral blood neutrophils are activated in patients with severe acne. ***Key words: neutrophils; T-cells; NK-cells; HLA Cw6.***

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The role of non-immunological mechanisms in the pathogenesis of acne is well accepted (1). The increasing evidence of immunological involvement, particularly in severe acne, includes reports of elevated antibody levels (2, 3) and cellular immunity to *Propionibacterium acnes* (2, 4) as well as increased numbers of circulating helper T-cells (5). Complement activation may also be involved, because patients with severe acne have high levels of complement fixing antibodies to *P. acnes* (6, 7). Observations on early acne lesions suggest that a release of chemoattractants and a subsequent influx of polymorphonuclear leukocytes (PMN) and lymphocytes are the initial inflammatory events (1). PMN functions have received attention (8–10), and a positive correlation between acne severity and PMN chemotaxis has been reported (11). There is also *in vitro* and *in vivo* evidence suggesting that isotretinoin, a drug used widely in the treatment of severe acne, produces significant anti-inflammatory effects by inhibiting monocyte and PMN chemotaxis (12).

In the present study we investigated the whole blood chemiluminescence (CL), lymphocyte subsets, natural killer (NK) cell activities and HLA antigens of patients with severe nodular acne (SNA) and acne fulminans (AF). Samples were taken from peripheral blood in the active phase of the disease and in remission.

MATERIAL AND METHODS

The 11 patients with SNA were 4 females aged 13–29, mean 22.2 years and 7 males aged 15–24, mean 19.8 years. The patients had papulopustules and several nodulocysts (>5 mm), and 3 of them also had a few ulcerations (Table I). The 4 patients with AF were males aged 15–19, mean 16.2 years. Three of them had fever >38°C, all had musculoskeletal pain and elevated ESR/C-reactive protein levels or leukocytosis (Table I), and the patients fulfilled the criteria of AF (13).

Before entering the study, 5 patients with SNA had received antibiotics (tetracyclin or cephadroxil). All the 4 patients with AF had also been treated with antibiotics (tetracyclin, cephalexin, ciprofloxacin or erythromycin) and one had also received isotretinoin. After entering the study, the patients with SNA were treated with prednisolone (initial dose 40 mg/d for one month) or isotretinoin (initial dose 0.7–1.0 mg/kg/d), both combined with erythromycin acistrate 800 mg/d. The patients with AF were treated with prednisolone (initial dose 50 mg/d) and erythromycin acistrate 800 mg/d. Moreover, one AF patient received isotretinoin throughout the study. Blood for the examinations was drawn in the active phase of the disease before the medication was started. The second sample was taken 18–52, mean 24.3, weeks later from the patients with SNA, and 18–45, mean 34, weeks later from the patients with AF, when all the patients had been without any medication for at least 2 weeks. At this time, 9 patients with SNA and all the 4 patients with AF were in clinical remission, but 2 SNA patients still had some acne lesions (Table I).

Chemiluminescence assay

The CL assay was carried out using whole blood as described earlier (14, 15) with some modifications. In brief, 80 µl of heparinized blood was mixed with 920 µl of phosphate buffered saline (PBS) and was allowed to stabilize at room temperature for 30 min. Phorbol myristate acetate (PMA, Sigma P 8139) was first dissolved in dimethylsulfoxide (DMSO) and then diluted with PBS to give the stimulating solution. Luminol was first dissolved in DMSO in dark and then diluted with PBS to give the responding solution. For the CL assay the blood PBS (100 µl) and luminol (800 µl) solutions were mixed and allowed to stabilize at room temperature for 20 min. Thereafter, PBS containing DMSO was added into the control tubes and PMA solution (100 µl) was added into the test tubes, and the CL measurements were started o immediately using an LKB Wallac luminometer 1251 at 37°C (LKB Wallac, Turku, Finland). The assay solutions (1000 µl) contained 8 µl whole blood, 0.33% of DMSO, luminol (5×10^{-4} M) and PMA (1×10^{-5} M). The results were plotted by using the computer connected to the luminometer and the PHAGO software (LKB Wallac, Turku, Finland). The highest (peak) value (millivolts, MV) and the integrated response (MV × min) up to 30 min were recorded. Every measurement included two control samples: one from a patient without a stimulating solution and the other from one of the four healthy persons constituting the laboratory personnel (aged 30–45 years). A plain background emission ranging from 0.64 to 1.51 mV was observed, but it did not differ between the patients and the controls. The correction based on neutrophil count involved dividing the expressed CL value with the neutrophil

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Table I. *Number of acne lesions and routine laboratory parameters in patients with severe nodular acne and acne fulminans*
CRP = C-reactive protein.

	Severe nodular acne		Acne fulminans	
	Active phase	Remission	Active phase	Remission
Papulopustules	36.4 (21.3)*	18.3 (37,8)**	177.8 (140.5)	6.5 (6.2)
Nodulocysts	16.4 (15.8)	1.2 (2.1)**	96.5 (13.4)	0.5 (1.0)
Leukocytes ($\times 10^9$/l)	7.9 (1.6)	5.8 (1.4)	13.9 (1.2)	4.9 (1.2)
ESR mm/h	10.7 (9.3)	6.8 (4.8)	46,8 (18.5)	5.5 (3.3.)
CRP mg/l	4.2 (3.0)	2.1 (2.5)	43.8 (55.5)	1.8 (2.9)

* Mean value (SD).
** Two patients with SNA were only in partial remission.

count; thus the presented value is CL response per 1×10^9 neutrophils/l. The quenching effect of erythrocytes was corrected by diluting the blood sample (1:125), so that it was not necessary to use a correction factor (16).

T-lymphocyte subsets and soluble interleukin 2 receptor (IL-2R)

Mononuclear cells were obtained from heparinized venous blood by Ficoll-Isopaque centrifugation. The cells were stained with monoclonal antibodies according to standard procedures, and the number of positive cells was quantified in flow-cytometry (FACScan, Becton Dickinson, Mountainview, CA, USA) as previously described (17). In two-colour analyses, CD4 or CD8 positive T cells were stained simultaneously with phycoerythrin (PE) conjugated monoclonal antibodies Leu 3a or Leu 2a (Becton Dickinson) and fluoresceinisothiocyanate (FITC) conjugated monoclonal anti HLA-DR antibodies (anti-DR, Becton Dickinson). FITC-conjugated Leu IIa antibody (Becton Dickinson) was used to stain CD16 positive NK cells. The percentage (%) of CD4, CD8 and CD16 positive cells was calculated from the total number of cells gated to the lymphocyte subpopulation and the proportion of HLA-DR positive cells from the total number of CD4 and CD8 positive cells. PE- and FITC-conjugated mouse IgG1 control antibodies were used to exclude non-specific binding.

The normal values, defined as mean +2 SD from a large series of healthy individuals, were the following: CD4 positive cells 36–54%, CD8 positive cells 27–39%, CD16 positive cells 11–20%, CD4/CD8 ratio 0.9–2.6, HLA-DR-positive CD4 and CD8 cells 0.9–2.2%.

IL-2R levels in the serum samples were determined using an enzyme immunoassay kit (T Cell Sciences Inc., Cambridge, MA, USA). Values exceeding 500 units/ml were considered positive.

NK cell functions

Preparation of effector cells. Venous blood samples (20 ml) were drawn into heparinized (20 U/ml) Venoject® tubes. Peripheral blood mononuclear cells (PBMC) were separated by gradient centrifugation using the Ficoll-Isopaque (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient. The PMBCs were washed twice with PBS (pH 7.4), after which non-adherent cells were first enriched by 1 h incubation on plactic tissue culture dishes at 37°C. The remainder of the adherent cells were removed by washing the PMBCs onto a 500 mg acid-treated nylon wool (Nycomed®, Bromma, Sweden) column. After 30 min incubation at 37°C, the non-adherent effector cells were eluted by PBS, pelleted by low-speed centrifugation and suspended into RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics (growth medium).

Preparation of target cells. K-562 target cells (1×10^6) were grown in growth medium and labelled with 250 **μCi of ^{51}Cr isotope (The Radiochemical Centre Amersham, Buckinghamshire, UK) for 1 h at 37°C.**

NK activity assay. A 4-h ^{51}Cr release assay was performed in triplicate in round-bottom microtiter plates (Nunc, Roskilde, Denmark) at 37°C. Various effector/target ratios (range 50:1 to 12.5:1) were used. The supernatants were harvested and counted with a Clinigamma® (LKB Wallac, Uppsala, Sweden) counter. The mean percentages of special lysis were calculated by the formula: Expressed counts per minute – Spontaneous counts per minute/Maximum counts per minute – Spontaneous counts per minute.

HLA-typing

HLA-typing was carried out using a standard two-stage microlymphocytotoxicity test (18) with immunomagnetic beads (Dynal A.S., Oslo, Norway) coated with monoclonal antibodies against either class I (HLA-A, -B, -C) or class II (HLA-DR) antigens. The results were read with a fluorescence microscope. The control HLA frequencies were from 188 healthy Finnish blood donors.

Statistics

The Wilcoxon matched pairs test was used for comparing the CL values in the active phase of the disease and in remission. HLA frequencies were analyzed by the χ^2 contingency test (with Yates' correction for 2×2 tables), and the corrected p-value (pc) was obtained by multiplying the original p-value with the number of HLA-antigens analyzed.

RESULTS

Chemiluminescence assay

The results are shown in Table II and Fig. 1. The serial samples in the active phase and in remission were obtained from 9 patients with SNA and from 2 with AF. The patients with SNA showed significantly higher peak CL values in the active phase compared to remission ($p = 0.004$, Table II). The CL values from the controls showed minimal variability when successive samples were examined.

The peak and the 30 min integrated CL values corrected on the basis of the neutrophil count were also significantly increased in the active phase, compared to remission in the patients with SNA (peak values mean 2.9 mV, SD 1.52 in the active phase and 1.52 mV, SD 0.7 in remission, $p < 0.05$, integrated values mean 47 mV, SD 24.8 in the active phase and 32 mV, SD 8.3 in remission, $p < 0.05$). The 2 patients with AF showed similar increases of the peak and integrated CL values in the active phase (Table II, Fig. 1).

In the active phase of the disease, the time required to reach the peak CL value ranged from 6 to 14 min, and the mean was

Table II. *Whole blood peak chemiluminescence results*[1] *in 9 patients with SNA, in 2 patients with AF and in 4 healthy controls*

Age/sex	Results of the patients		Results of the controls[2]	
	Active phase	Remission	Active phase control	Remission control
Severe nodular acne				
F/29	21.0 (8)*	8.7 (6)	6.1 (12)A	6.6 (10)B
F/13	12.6 (14)	2.7 (12)	6.7 (12)A	7.4 (10)B
M/20	9.6 (6)	3.5 (14)	6.7 (6)B	3.5 (12)C
M/18	14.6 (6)	4.5 (16)	5.1 (10)C	5.0 (12)C
M/24	5.2 (10)	3.0 (14)	4.7 (10)C	6.7 (12)C
M/21	13.0 (12)	10.2 (16)	4.8 (10)C	3.6 (14)B
M/19	7.6 (10)	4.6 (10)	8.0 (12)B	9.0 (14)B
F/27	13.6 (6)	6.8 (12)	4.9 (19)B	4.8 (6)C
M/15	45.3 (14)	9.4 (16)	6.7 (12)B	4.4 (14)C
Mean	15.8 (9.6)	5.9 (12.9)	6.0 (11.4)	5.7 (11.6)
SD	11.9	2.9	1.2	1.9
Acne fulminans				
M/15	44.2 (14)	2.1 (16)	5.5 (14)C	3.7 (13)C
M/16	39.4 (10)	8.4 (8)	5.5 (14)D	4.8 (10)C
Mean	41.8 (12)	5.2 (12)	5.5 (14)	4.2 (11)
SD	3.4	4.5	0	0.8

[1] The values are not corrected on the basis of neutrophil count.
[2] Four healthy female controls (A, B, C and D).
*Peak CL/mV and (time/min) required to reach the peak value.

9.6 min in patients with SNA, 12 min in patients with AF and 11.4 min in controls (Table II). In remission, the corresponding mean times were 12.9 min in patients with SNA, 12 min in patients with AF and 11.6 min in controls. Thus, the kinetics of the CL curves remained similar in the patients and in the controls and were constant irrespective of the activity of the disease.

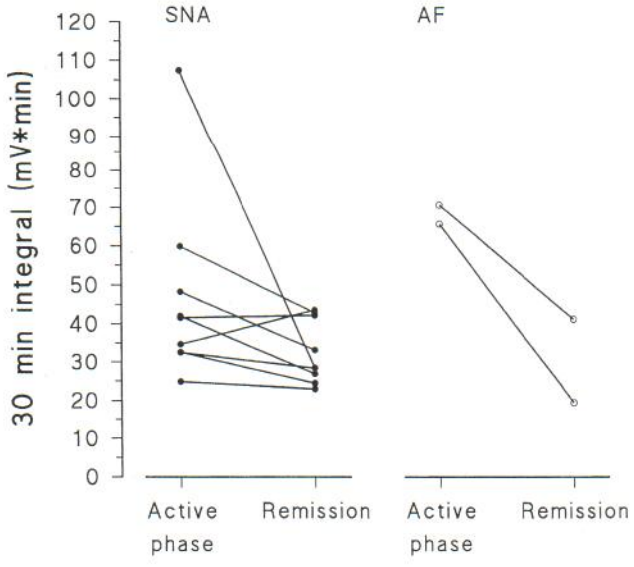

Fig. 1. Thirty minutes integrated CL values in 9 patients with SNA and in 2 patients with AF are shown in the active phase of the disease and in remission. One SNA patient, who had a very severe form of the disease, also showed an exceptionally high CL value (107 mV × min) in the active phase.

T-lymphocyte subsets and soluble interleukin 2 receptor

The results are shown in Table III. In patients with SNA the percentage of CD4, CD8 and CD16 positive cells was within the normal range and showed no significant changes between the samples taken in the active phase and in remission. Only one SNA patient, who did not achieve complete remission, showed a decrease in the CD4 cell count (from 51% to 39%), and two SNA patients had low CD4/CD8 ratios in the active phase (0.80 and 0.79) and also in remission. No marked changes were observed in HLA-DR positive CD4 and CD8 cells.

In the patients with AF, a slight increase occurred in the mean percentage of CD4 (from 41 to 48%) and the CD8 positive cells (from 33 to 36%) from active phase to remission. In contrast, the HLA-DR positive CD4 cells showed a slight decrease (from 16% to 10%), but the mean percentage of CD16 positive cells remained at the same level.

The mean soluble IL-2R levels in the active phase of the disease were 367 units/ml in patients with SNA and 586 units/ml in patients with AF. In remission, the values were 297 units/ml and 356 units/ml, respectively. One patient with SNA and one with AF showed clearly elevated levels of IL-2R (1022 units/ml and 1058 units/ml, respectively) in the active phase of the disease, but in both cases the levels came down to normal when the patients were in remission (433 units/ml and 399 units/ml, respectively).

NK cell functions

An increasing trend was found in all the three effector:target cell ratios when the values in the active phase were compared to those in remission (Table III).

HLA-typing

Four patients with SNA and one patient with AF carried HLA Cw6 antigen. The frequency of the patients (33%) was significantly increased when compared to the corresponding frequency of the controls (5%, $p = 0.0005$, $pc = 0.015$). The frequencies of HLA DR3 (7%) and B27 (7%) as well as the frequencies of the other HLA antigens were at the same level as in the healthy controls.

DISCUSSION

Inflamed acne lesions are characterized by dermal infiltration of PMNs in response to chemoattractants produced possibly by bacteria in occluded sebaceous ducts (19). The functions of the PMNs in peripheral blood have also been investigated in patients with acne. Wahba in 1978 (9) and Rebora et al. in 1979 (8) studied PMN chemotaxis, migration and phagocytosis in the active phase of acne conglobata in 18 patients but did not find any major abnormalities. Christophers & Schröder (10) investigated PMN responses in a 40-year-old woman with severe relapsing acne. During the course of the disease, they detected a transient decrease in several C5a-induced PMN cell functions, whereas the response to N-formylmethionyl peptides was normal. Gould et al. (11) measured the intracellular killing power of PMNs and the chemotactic response of PMNs and monocytes in patients with acne vulgaris. They detected a positive correlation

Table III. *T-lymphocyte subsets and the results of NK cell functions in patients with severe nodular acne and acne fulminans*

	Severe nodular acne				Acne fulminans			
	Active phase		Remission		Active phase		Remission	
T-cell subsets	Mean percentage (SD)							
Helper cells (CD4)	45	(9.3)	44	(8.0)	41	(9.0)	48	(9.0)
Suppressor cells (CD8)	36	(7.2)	38	(5.0)	33	(8.3)	36	(7.0)
TH/TS-ratio	1.2	(0.33)	1.2	(0.34)	1.4	(0.69)	1.4	(0.59)
DR^+ Helper cells	1.9	(1.0)	1.9	(1.0)	3.2	(0.8)	2.3	(1.1)
DR^+ Suppressor cells	2.4	(1.2)	2.7	(1.1)	3.2	(1.0)	3.1	(0.7)
NK cells (CD16)	16	(5.3)	18	(6.1)	22	(8.8)	21	(7.4)
Natural killer-cell functions								
Effector:target cell ratio	Mean percent lysis (SD)							
50:1	38	(16.7)	43	(15.4)	28	(14.2)	29	(15.6)
25:1	31	(15.5)	36	(15.4)	22	(7.3)	28	(19.7)
12.5:1	24	(15.4)	28	(15.3)	18	(8.6)	25	(21.4)

between acne severity and PMN chemotaxis, but no enhanced intracellular killing could be observed in the nitroblue tetrazolium test.

We investigated the reactive oxygen production of whole blood by a luminol-dependent CL assay in patients with SNA. When the values in the active phase of the disease were compared to those in remission, the former were found to be significantly higher. A similar trend was also seen in 2 patients with AF.

CL refers to the emission of light by chemical reactions. The reactions are usually oxidative, such as those in the respiratory burst of neutrophils (20). When measured from whole blood, CL is affected by the number of monocytes and neutrophils per sample, by humoral factors, and by the quenching of erythrocytes (16). However, the response induced by monocytes is markedly lower than that induced by neutrophils, and a correction based on the neutrophil count is therefore appropriate (21). We also diluted the blood samples (1:125) to minimize the quenching effect of erythrocytes. It is obvious that the increased CL values in the active phase were due to the increased production of reactive oxygen species by PMNs and not only to the increased number of PMNs, because the CL values corrected on the basis of the neutrophil count also showed significant changes. On the other hand, the variability of the CL responses between the samples taken from the controls at different times was quite small. These findings suggest that the metabolic burst of whole blood, mostly PMNs, is activated in severe acne. This burst may be due to the priming of neutrophils by various inflammatory mediators, such as cytokines or components produced by *P. acnes*. In the active phase, one patient with SNA showed equally high CL values as the patients with AF. In this patient the acne responded poorly to the treatment and turned into ulcerative acne. In agreement with our findings on severe acne, high CL values have also been reported by De Simone et al. (22) in patients with active psoriasis.

Retinoids and tetracycline have been reported to affect neutrophil functions *in vivo* and *in vitro* (12, 23–25). Esterly et al. (25) showed that tetracycline has a marked inhibitory effect on neutrophil chemotaxis both in patients with acne and in healthy controls. Norris et al. (12) similarly found isotretinoin treatment to produce significant inhibition of neutrophil and monocyte chemotaxis in patients with cystic acne. Contrary to this, enhanced CL responses have been reported in isotretinoin-treated patients with acne (24). In our study, several patients with SNA received isotretinoin, but the samples were taken before this treatment, suggesting that the increased CL values were not due to the therapy, but to the acne inflammation. Some of the patients also received prednisolone, but this medication was discontinued at least 2 weeks before the second blood sample was obtained. Corticosteroids at high concentrations have been reported to inhibit the production of reactive oxygen species *in vitro* (25).

The present results in patients with severe acne did not reveal any abnormalities in the T-lymphocyte subsets either in the active phase of the disease or in remission. Transient increases in the number of CD4 cells have been described in patients with acne (5) and during isotretinoin treatment (26). This drug has been reported to produce a reduction or an increase in the number and activity of NK cells (27, 28). In the present patients, the NK cell numbers and activities were normal, and hence we suggest that the immunologically active cells of peripheral blood remain at normal levels in severe acne.

Genetic factors may also be important, as we found that HLA Cw6 was significantly increased in our patients with acne. HLA Cw6 is known to be associated with psoriasis, a disease where PMNs are also involved in the pathogenesis (29).

In conclusion, the present CL results suggest that the PMNs are in an activated condition capable of releasing the reactive oxygen species in patients with severe acne. In contrast, we did not find any marked alterations in the percentages of T-cell subsets or NK cells or their activities. The frequency of HLA Cw6 was significantly increased, suggesting that genetic factors may be involved in severe acne.

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