Clin Exp Immunol 1993; 92:274–283

Local and systemic activation of the whole complement cascade in human leukocytoclastic cutaneous vasculitis; C3d,g and terminal complement complex as sensitive markers

H. DAUCHEL, P. JOLY*, A. DELPECH†, E. THOMINE‡, F. SAUGER§, X. LE LOET¶, PH. LAURET*, F. TRON†, M. FONTAINE & J. RIPOCHE *INSERM Unité 78, Bois-Guillaume, *Department of Dermatology, †Immunology Laboratory, ‡Department of Anatomo-Pathology, §Biochemistry Laboratory and ¶Department of Rheumatology, University Hospital, Rouen, France*

(Accepted for publication 8 January 1993)

SUMMARY

We have studied complement activation both in plasma samples and in lesional skin from patients with leukocytoclastic cutaneous vasculitis (LCV). Enzyme immunoassay (EIA) quantification of the complement activation markers, C3d,g and the terminal complement complex (TCC) in plasma, showed that their levels were significantly increased in 66% and 55% of the patients, respectively ($n=29$) compared with healthy controls, whereas the standard measurements of C3, factor B, C1q, C4 and C2 were generally within normal range. Elevations of C3d,g and TCC levels in plasma were significantly correlated. Importantly, a significant correlation was found between the severity of the vasculitis and both C3d,g and TCC plasma levels. Immunofluorescence studies of skin biopsy specimens demonstrated simultaneous presence of perivascular dermal deposits of C3d,g and TCC in lesional skin from 96% and 80% respectively of the patients ($n=25$). There was a significant correlation between the intensity of the deposits of both markers. Clusterin, a TCC inhibitory protein, was always found at the same sites of perivascular TCC deposits. Immunofluorescence studies at the epidermal basement membrane zone (BMZ) revealed in each case deposits of C3d,g which were accompanied by TCC deposits in 52% of the biopsy specimens. These data demonstrate that there is a local and systemic activation of the whole complement cascade in human LCV. The presence of both C3d,g and clusterin-associated TCC perivascular deposits suggests an intervention of a regulatory mechanism of local complement activation in LCV. Finally, measurement of plasma C3d,g and TCC appears to be a sensitive indicator of systemic complement activation and disease severity in LCV.

Keywords endothelium inflammation terminal complement complex clusterin immune complex

INTRODUCTION

Vasculitides refer to a group of diseases which have in common histological features of inflammation and lesions of blood vessel walls [1]. In leukocytoclastic cutaneous vasculitis (LCV), polymorphonuclear neutrophils, in a pyknotic form of nuclear fragmentation (leukocytoclasis), are predominantly found within and around the wall of the dermis small blood vessels. Blood vessel damage in LCV is generally thought to be mediated via the formation of antigen–antibody complexes, their subsequent deposition within the vessel walls and then the local activation of the complement system [2,3]. Immunofluorescence studies have supported this hypothetical mechanism of immune complex and complement intervention in LCV. Deposits of immunoglobulins of IgG, IgM and IgA classes have been detected in early biopsied lesional skin within and around the involved dermis vessel walls, colocalizing with complement component C3 deposits [4,5]. In addition, patients with LCV associated with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) or mixed cryoglobulinaemia, have reported plasma hypocomplementaemia, as revealed by measurement of native individual complement components. However, these measurements often gave inconsistent results and failed to account for the mechanism of hypocomplementaemia, synthesis, catabolism or consumption [6].

More recently, *in situ* detection or quantification in biological fluids of complement activation products such as the C3d,g, Ba and C4d fragments or the anaphylatoxins C3a, C4a and C5a, or the terminal complement complex (TCC), has been shown to be a more reliable approach to assessing complement activation

Correspondence: H. Dauchel, INSERM U-78, BP 73, F-76233 Bois-Guillaume Cedex, France.

[6]. These complement activation products and in particular C3d,g and TCC as markers of initial and terminal complement activation were found to be valuable plasmatic indicators of disease activity in SLE and in RA [7–10]. Their potential interest in LCV has not yet been investigated. Recent immunofluorescence studies showed that TCC perivascular deposits in lesional skin of LCV could be found, colocalizing with immunoglobulin and C3 deposits [5,11]. The TCC was found on the surface of endothelial cells of the dermal vessels and on infiltrating neutrophils [12]. These results were another indication that complement activation is directly involved in the vascular damage in LCV. However, a complete picture of complement activation, including markers of early and late phase activation, is still lacking.

Immunofluorescence studies have found a number of endothelial cell membrane-associated inhibitors of complement activation, such as decay-accelerating factor (DAF, CD55) [13], membrane cofactor protein (MCP, CD46) [14] and CD59 (protectin or HRF20) [15] on dermal vessels. These findings suggest that endothelium would be relatively resistant to direct attack by homologous complement and that vessel damage in LCV may at least in part be caused by TCC-induced proinflammatory non-lethal effects such as release of arachidonic or oxygen-active metabolites by endothelial cells [16]. In this regard, recent immunofluorescence studies of skin and renal biopsies in human glomerulonephritis and in Henoch–Schönlein purpura showed that TCC deposits are at least partially in an inactive form which contains the inhibitory S-protein (vitronectin) [11] or both S-protein and the newly discovered SP-40,40 protein [17–19], also called clusterin, sulphated glycoprotein-2 (SGP-2), and complement lysis inhibitor (CLI) [20]. Concerning LCV, it is not known whether or not TCC is in a cytolytically active form in the cutaneous deposits.

The aim of this study was to evaluate: (i) the participation of the whole cascade of complement in the pathogenesis of LCV lesions using C3d,g and TCC as markers of early- and late-phase activation respectively, both in plasma samples and on skin biopsies; (ii) the presence of the TCC-inhibitory protein SP-40,40 in cutaneous deposits in LCV; (iii) the usefulness of C3d,g and TCC measurement for the assessment of disease severity in LCV.

PATIENTS AND METHODS

Patients

Twenty-nine patients (13 females, 16 males, mean age 62 years, range 18–90 years), seen in the Departments of Dermatology and Rheumatology of the University Hospital of Rouen (France), with clinical and histological features of LCV, were included in this study over an 18-month period. This study was conducted after approval by the Rouen School of Medicine ethical committee. For 25 patients, a skin biopsy was performed on lesional skin and divided in two parts: one for histological diagnosis of the vasculitis, the other for specific immunofluorescence studies. Lesional skin was always obtained during an active phase of the disease.

Disease activity

Grades of disease activity were defined by the following three parameters: (i) clinical extent of the lesions, scored 1 if only legs and/or feet were involved, and 2 if trunk, face and/or arms were also involved; (ii) clinical evidence of cutaneous necrosis was scored 0 if absent and 1 if present; (iii) clinical or biological features of systemic participation were scored 1 if one of the following manifestations was present: renal injury, fever, arthralgia/myalgia, inflammatory syndrome, and 2 if there were more than one of them. According to these criteria, patients were classified into three groups with grades of increasing disease activity. Group I consisted of eight patients with a score 1 or 2. All of them had drug-induced LCV and no clinical features of systemic participation. Group II consisted of 13 patients with a score 3. In this group, five of the patients had drug-induced or infectious disease-associated LCV and had no symptom of systemic participation, whereas the eight other patients had extracutaneous clinical features (rheumatoid vasculitis, $n=2$; drug-induced LCV, $n=5$; LCV of unknown etiology, $n=1$). Group III consisted of eight patients with a score 4 or 5. All of them had clinical features of systemic participation and two patients died during the course of the disease. In this group, the LCV was associated with SLE ($n=1$), rheumatoid vasculitis ($n=3$), cryoglobulinaemia ($n=1$), a suspected tumoral process ($n=1$) or was drug-induced ($n=2$).

Controls

Control group 1 included, after formal consent was given, 30 healthy individual donors for plasma complement component measurements and five for skin biopsy immunofluorescence studies. A second group of controls (control group 2) consisted of lesional skin specimens and plasma samples from six subjects with various inflammatory skin disorders other than LCV or autoimmune bullous skin diseases: eczema ($n=3$), mastocytosis ($n=1$), polymorphous light eruption ($n=1$), or psoriasis ($n=1$). All these subjects were in an active phase of the disease.

Histopathological diagnosis

The first part of the biopsy specimen obtained from patients with LCV was fixed in formaldehyde and sections of paraffin-embedded tissue were stained with haematoxylin, eosin and safranin. Criteria for histopathological diagnosis of LCV were the presence within and around small blood vessel walls of the superficial or reticular dermis of (i) polymorphonuclear neutrophils with fragmented nuclei (leukocytoclasis), (ii) fibrin deposition, (iii) erythrocyte extravasation. Necrotic changes in the blood vessel walls were also looked for [1,2].

Immunofluorescence procedures

The second part of each biopsy specimen from patients with LCV and control groups 1 and 2 was snap-frozen in liquid nitrogen and stored at $-70°C$ until use. Cryostat sections (3 μm) of the specimens were air dried for 30 min at 25°C, rehydrated with PBS for 5 min at 25°C and then overlaid with FITC-conjugated antisera (direct immunofluorescence) or with unlabelled antibodies (indirect immunofluorescence) for 30 min at 25°C. For indirect immunofluorescence, sections were further incubated, after three washes in PBS 7 min each, with FITC swine anti-rabbit or with FITC rabbit anti-mouse antisera (Dakopatts, Copenhagen, Denmark). Commercial monospecific FITC-conjugated antibodies were used to detect deposition of IgG, IgA, IgM, components C3, C4 (1:5 in PBS; Behr-

ingwerke, Marburg Lahn, Germany), C1q (1:5 in PBS; Nordic-TEBU, Le Perray-en-Yvelines, France) and fibrinogen (1:20 in PBS; Selenu-Eurobio, Paris, France). Two mouse MoAbs to neoantigens of human TCC were used (1:20 in PBS). One was from Dakopatts. The other one was a generous gift from Dr S. Bhakdi (Mainz, Germany). It shows strong cross-reactivities with neoantigens of rabbit TCC [21]. A mouse MoAb to human clusterin (G7 MoAb, 1:50 in PBS) (generous gift from Dr B. F. Murphy, Melbourne, Australia [17]) and a rabbit polyclonal monospecific antibody to human C3d,g/C3d (1:100 in PBS; Dakopatts) were used. The sections were examined by two investigators with a fluorescence microscope (Leitz). The observations were semiquantified as follows: −, negative; +, traces; ++, moderate; +++, intense deposition. Micrographs were taken with a high-speed Ektachrome ASA 400 film.

Blood collection

Blood samples from patients with LCV, control groups 1 and 2 subjects, were drawn on EDTA (final concentration 10 mM) and immediately centrifuged (800 ***g***, 10 min, 4°C). The plasma-EDTA were immediately frozen at −70°C in 0·2-ml aliquots until assayed. Each aliquot was discarded after a single use. Blood samples were drawn from each patient with LCV and 1 control group 2 subject at the same time the cutaneous biopsies were performed.

Measurement of native complement proteins

Levels of C3, C1q, C4, and factor B in plasma were measured by laser nephelometry (Behringwerke) according to the manufacturer's instructions. Levels of C2 and CH_{50} in plasma were assayed using standard haemolytic techniques [22].

Measurement of complement activation products

Levels of TCC in plasma samples were quantified by enzyme immunoassay (EIA) with a commercial kit (Quidel, San Diego, CA) according to the manufacturer's instructions. Quantification of C3d,g was performed by EIA. Briefly, polystyrene plates (CML, Nemours, France) were coated with a rabbit polyclonal monospecific antibody anti-C3d,g/C3d (Dakopatts), diluted 1:1000 in 0·1 M $NaHCO_3$ pH 9, 2 h at 37°C. C3d,g standard was derived from a pool of 100 normal human sera, by incubation with inuline (Sigma via Eurobio, Paris, France) 3 mg/ml, 2 h at 37°C, according to the method described [22]. This inuline-treated serum was taken as the 100% activation reference serum. To eliminate the remaining C3, 100 μl of each sample and standard were allowed to precipitate for 15 min at 25°C with 20 μl of PEG-6000 (Sigma via Eurobio) (25% (w/v) in NaCl 0·15 M) and 40 μl of rabbit polyclonal anti-C3c antibody (Dakopatts) as described [23]. After centrifugation for 2 min at 12000 ***g***, the C3d,g-containing supernatants were diluted in PBS containing 0·1% bovine serum albumin (BSA) as carrier protein. The 100% activation reference serum was routinely diluted two-fold from 1:500 to 1:64000, whereas the plasma samples were diluted 1:80 and 1:160. One hundred microlitres of the diluted plasma samples or standard serum were added in duplicate to the wells and plates were incubated for 2 h at 37°C. Bound C3d,g were detected by incubation with peroxidase-conjugated anti-C3d antibody (Dakopatts) diluted 1:1000 in PBS containing 0·1% BSA 2 h at 37°C. Results were expressed as percentage of complement activation by comparison with the reference serum. Finally, data points on figures represent the mean values between the results of duplicate measurements at intervals of a few days. C3d,g concentration in the 100% reference serum was determined using a purified preparation of C3d (kind gift from Dr A. Ischenko, St Petersburg, Russia). It was found to be 640 μg/ml. Therefore, C3d,g levels in plasma samples expressed as percentage of complement activation from reference serum could be converted into μg/ml by multiplying by a factor of 6·4.

Statistical analysis

Non-parametric statistical methods were employed using the solo program (BMDP, Los Angeles, CA). The Mann–Whitney rank sum test was used to compare different groups. The Spearman's rank correlation coefficient (r_s) was used to examine correlation between different indicators. P values $\leq 0{\cdot}05$ were considered significant.

RESULTS

High incidence of perivascular TCC and C3d,g dermal deposits in LCV

Table 1 summarizes the immunofluorescence analysis of lesional skin biopsy specimens from 25 patients with LCV. In 24 of them (96%), C3d,g perivascular deposits were found. The fluorescence gave a thick and linear staining pattern, as shown in Fig. 1a. In 20 of these 24 C3d,g-positive biopsy specimens, TCC perivascular deposits were demonstrated in a fine and granular staining pattern (Fig. 1b), in the same dermis area as C3d,g deposits. Moreover, linear regression analysis revealed a significant positive correlation between TCC and C3d,g perivascular fluorescence intensity ($r_s = 0{\cdot}56$, $P < 0{\cdot}01$, correlation plot not shown). Therefore, there were signs of activation of the whole cascade of complement in 80% of the lesional skin biopsies ($n = 25$). Clusterin was looked for in 11 biopsy specimens. It was found as perivascular deposits in seven of them and always associated with TCC perivascular deposits (Fig. 1c). Clusterin deposits were not observed in the absence of TCC. Perivascular deposits of immunoglobulin were found in 56% of the 25 biopsy specimens. IgM and surprisingly IgA (48% of the specimens), were the more frequent immunoglobulin classes deposited in the vessel walls of skin lesions. IgG deposits were found in four of the 25 specimens, only with IgA and IgM. Immunoglobulin deposits, when detected, were always associated with C3d,g deposits, and a significant positive correlation between the intensity of the two deposits was observed ($r_s = 0{\cdot}53$, $P < 0{\cdot}01$, correlation plot not shown). In control groups 1 and 2, no perivascular deposit was found in the dermis of the biopsy specimens.

We also looked for immunoglobulin and complement activation marker deposits at the epidermal basement membrane zone (BMZ) of the skin biopsy specimens of 25 patients with LCV. In 22 of them (88%), moderate or intense C3d,g deposits were observed. The fluorescence gave a granular continuous staining pattern (Fig. 2a). Of interest was the finding of TCC deposits at the BMZ in 52% of the specimens. They were found in a fine granular discontinuous staining pattern (Fig. 2b), and always in association with C3d,g deposits. In contrast with perivascular findings, no deposits of the clusterin could be demonstrated at the BMZ. We also observed immunoglobulin deposits at the BMZ in three biopsy specimens. Two came from SLE patients and the immunoglobulin deposits correspond to

Table 1. Immunofluorescence findings of complement, immunoglobulin and fibrinogen perivascular deposits in lesional skin biopsy specimens from 25 patients with leukocytoclastic cutaneous vasculitis (LCV).

Patient no.	IgA	IgM	IgG	C3dg	TCC	Clusterin	C3	Clq	C4	Fib
1	−	−	−	+	+	+	+	+	+	−
2	++	++	−	+++	++	++	+++	+	+	++
3	++	+++	++	+++	++	++	+	−	−	++
4	+	−	−	+++	−	−	+++	−	−	++
5	−	−	−	−	−	−	−	−	−	++
6	−	−	−	+	−	ND	−	−	−	+
7	−	−	−	+	−	−	−	−	−	−
8	+	+	+	++	−	−	−	+	+	+
9	++	+++	++	+++	++	++	++	+	+	++
10	−	++	−	++	++	++	++	−	−	++
11	++	++	−	++	++	++	+	+	+	+++
12	−	+	−	+	+	ND	+	−	−	+
13	−	−	−	++	++	ND	+	−	−	++
14	+	−	−	+++	++	ND	+	−	−	++
15	−	−	−	+	+	ND	−	−	−	−
16	−	−	−	++	+	ND	+	−	−	++
17	+	++	++	+++	+++	ND	++	+	+	++
18	−	−	−	++	++	ND	−	−	−	++
19	−	−	−	+	++	ND	−	−	−	−
20	+	++	−	+++	++	ND	+++	+	+	+++
21	−	−	−	+++	+++	ND	−	−	−	+
22	++	−	−	+++	+++	ND	−	−	−	++
23	++	+	−	++	++	ND	++	−	−	++
24	−	−	−	+++	+	ND	+	+	+	+++
25	+++	+++	−	++	++	++	++	++	++	+

ND, Not done; −, negative; +, traces ; ++, moderate; +++, intense deposits; Fib, fibrinogen.

the typical lupus 'band'. The third was obtained from a patient with a rheumatoid vasculitis, and contained IgG, IgA and IgM. In control groups 1 and 2, a continuous deposit of C3d,g at the BMZ was invariably observed, whereas no TCC or immunoglobulin deposits could be evidenced.

C3d,g and TCC are sensitive markers of systemic complement activation in LCV

Figure 3 shows that the levels of native complement proteins C3, factor B, C1q, C4 and C2 were in almost all cases within normal range. Only one patient had low levels of C1q, C4, C2 and non-detectable CH_{50}, indicating complement activation through the classical pathway. In contrast to these standard measurements of native complement proteins, quantification of complement activation markers in plasma showed that C3d,g and TCC levels were both significantly increased in 65·5% and 55%, respectively, of the patients with LCV, compared with control group 1 ($P<0·0001$, $n=29$; Figs 4a,b). Particularly, the patient with severe breakdown of CH_{50}, C1q, C4 and C2, had extremely increased levels of C3d,g (17%) and TCC (155 ng/ml) in plasma, indicating a great consumption of C3 and the involvement of the final pathway of complement. None of the subjects in control group 2 showed evidence of complement activation in plasma, and their levels of both C3d,g and TCC were significantly lower than those in patients with LCV ($P<0·001$; $P<0·0001$). As shown on the correlation plot in Fig. 5, there was a positive correlation between C3d,g and TCC levels in plasma in patients with LCV ($r_s=0·64$; $P<0·001$), indicating systemic activation of the whole cascade of complement. Surprisingly, CH_{50} levels in plasma appeared to be significantly increased in 65% of the patients ($P<0·0001$; Fig. 3).

Correlation between levels of C3d,g and TCC in plasma and disease severity

Grades of disease activity were scored, as described in Patients and Methods. Patients were accordingly assigned to three groups with increasing grades of disease activity. Figures 6a,b show C3d,g and TCC levels in plasma in the three groups of patients. A positive correlation between both C3d,g and TCC levels in plasma and disease activity was observed ($r_s=0·66$, $P<0·001$ and $r_s=0·44$, $P<0·02$).

Lack of correlation between local and systemic complement activation

In order to determine whether systemic activation of complement reflected the local activation, we compared C3d,g and TCC levels in plasma in two groups of patients with LCV having respectively no or traces and moderate or intense perivascular deposits of C3d,g and TCC on lesional skin biopsies. Figures 7a,b show an increase of the mean values of both C3d,g and TCC levels in the group of patients with moderate or intense perivascular deposits, compared with the group of patients with

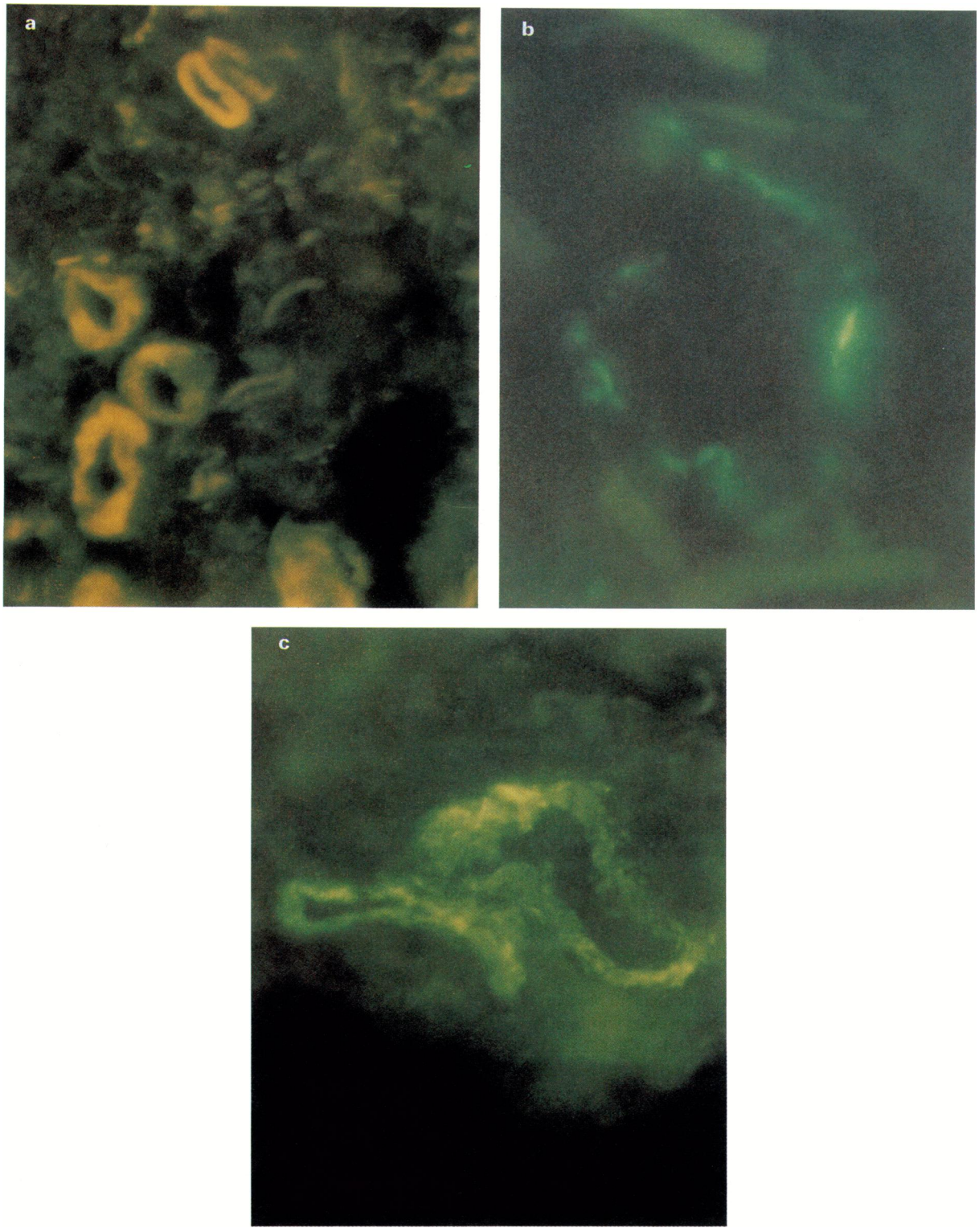

Fig. 1. C3d,g (a), terminal complement complex (TCC) (b) and clusterin (c) perivascular deposits in dermal vessels of lesional skin biopsy specimens from patients with leukocytoclastic cutaneous vasculitis (LCV). Original magnification × 1000.

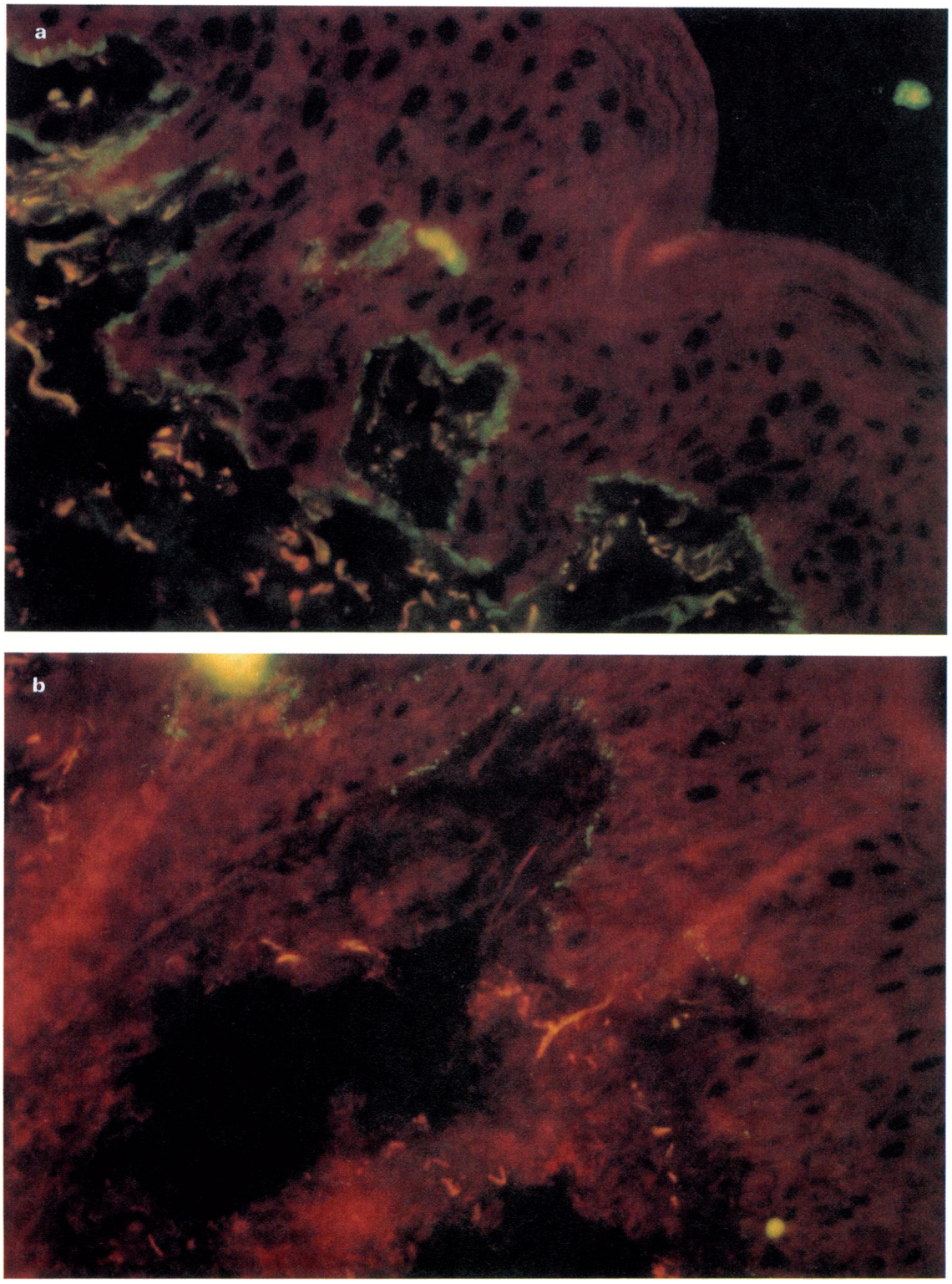

Fig. 2. C3d,g (a) and terminal complement complex (TCC) (b) deposits at the epidermal basement membrane zone in lesional skin biopsy specimens from patients with leukocytoclastic cutaneous vasculitis (LCV). Original magnification ×400.

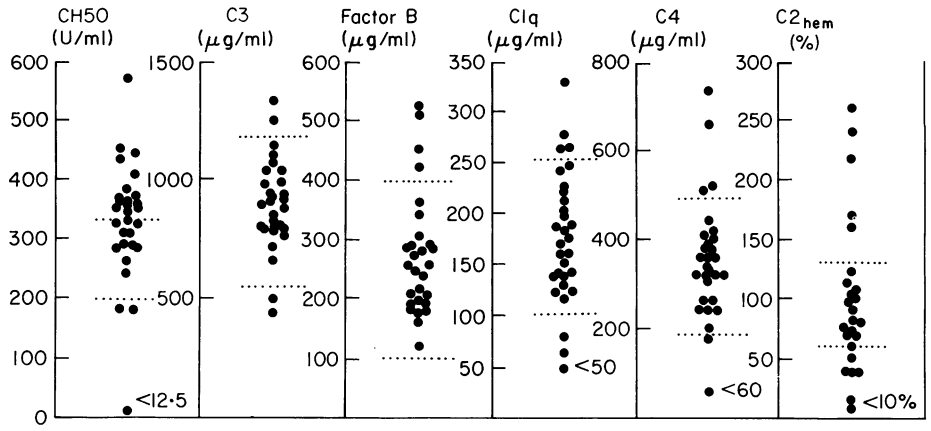

Fig. 3. Plasma complement measurements : CH_{50}, C3, factor B, C1q, C4, C2. Number of patients with leukocytoclastic cutaneous vasculitis (LCV): 29. Horizontal dotted lines indicate the lower and upper limits of normal.

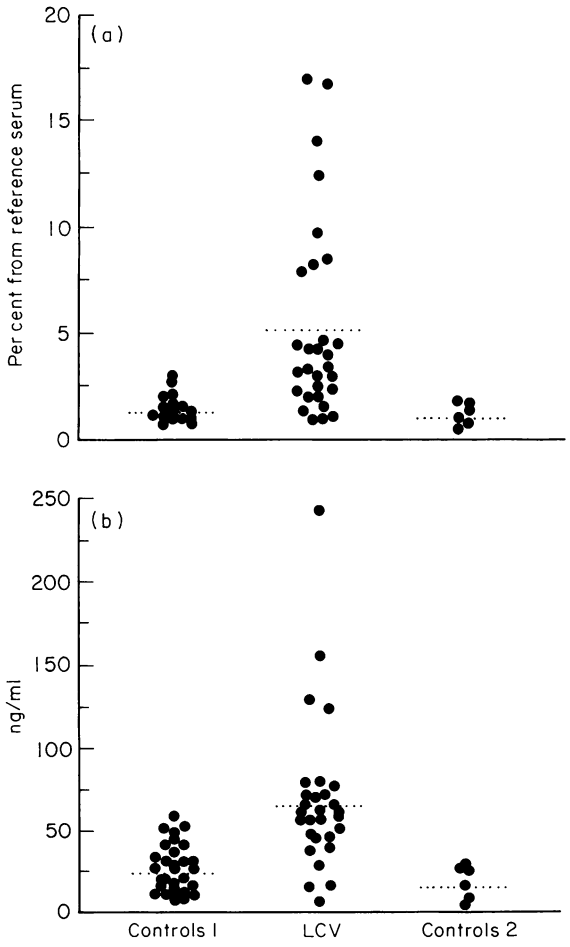

Fig. 4. C3d,g (a) and terminal complement complex (TCC) (b) levels in plasma from healthy donors (controls 1, $n=30$), patients with leukocytoclastic cutaneous vasculitis (LCV) ($n=29$) and subjects with other inflammatory cutaneous diseases (controls 2, $n=6$). Dotted lines represent mean values.

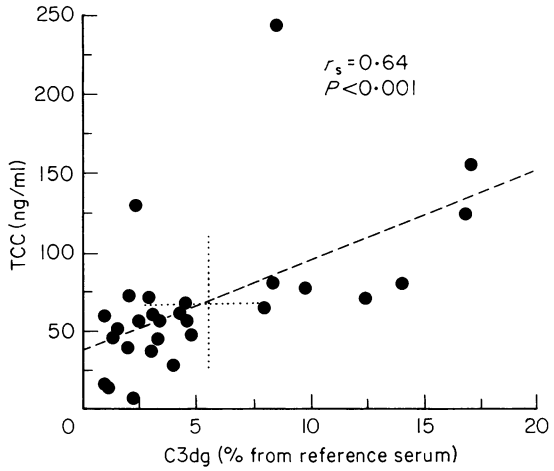

Fig. 5. Correlation plot of C3d,g and terminal complement complex (TCC) levels in plasma from patients with leukocytoclastic cutaneous vasculitis (LCV). Horizontal and vertical dotted lines represent mean values.

no or only traces of these deposits. However, regression analysis did not demonstrate significant positive correlations between the levels in plasma and the intensity of cutaneous deposits ($r_s=0{\cdot}24$, $P<0{\cdot}2$ for C3d,g; $r_s=0{\cdot}11$, $P<0{\cdot}9$ for TCC).

DISCUSSION

This study brings conclusive evidence of activation of the whole cascade of the complement system both locally, in lesional skin, and in plasma from patients with LCV. A number of important findings can be demonstrated: (i) both the perivascular space and the epidermal BMZ were involved locally and both early and terminal pathway of the complement were activated; (ii) when analysed, clusterin was systematically associated with TCC perivascular deposits; (iii) there was a high correlation between deposits of complement activation product C3d,g and immunoglobulin deposits, which were in the form of IgA deposits in half of the patients; (iv) C3d,g and TCC levels were increased in plasma of patients with LCV compared with

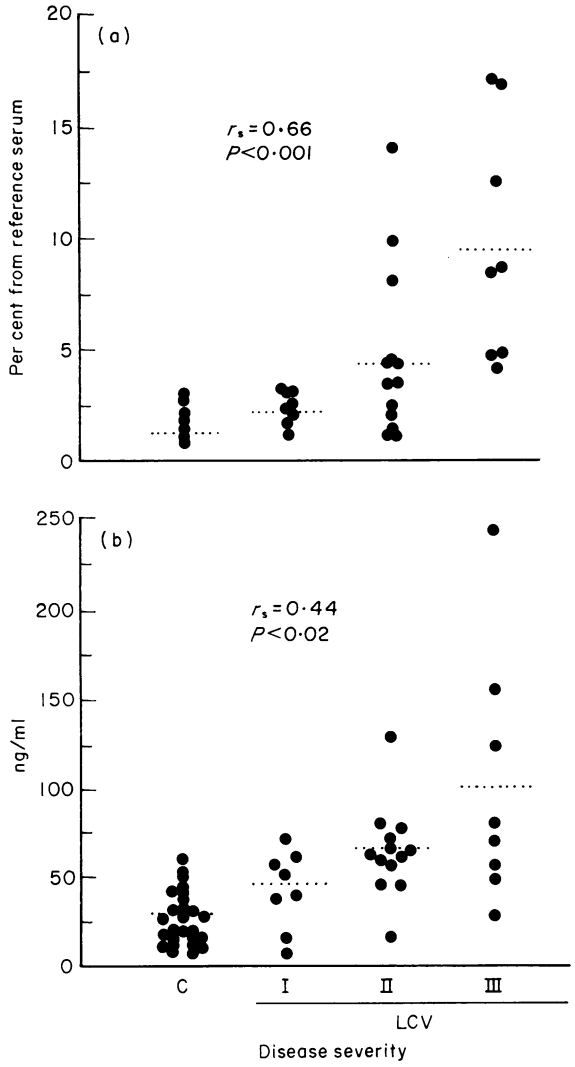

Fig. 6. C3d,g (a) and terminal complement complex (TCC) (b) levels in plasma from healthy donors (controls 1, $n = 30$) and subgroups of patients with increasing grades of leukocytoclastic cutaneous vasculitis (LCV) activity (see Patients and Methods for details). Dotted lines represent mean values.

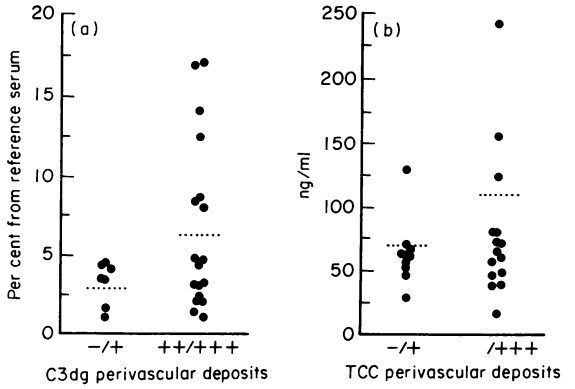

Fig. 7. C3d,g (a) and terminal complement complex (TCC) (b) levels in plasma in subgroups of patients with leukocytoclastic cutaneous vasculitis (LCV) with increasing intensity of cutaneous perivascular C3d,g and TCC deposits. Dotted lines represent mean values. −, negative; +, traces; + +, moderate; + + +, intense deposits.

healthy controls, whereas standard measurements of complement native proteins failed to detect this abnormal activation of complement; (v) levels of C3d,g and TCC in plasma were correlated with the severity of the vasculitis.

Immunofluorescence studies revealed for the first time perivascular deposits of both C3d,g and TCC (in 96% and 80% of the patients respectively) in the lesional skin of patients with LCV. Since the polyclonal anti-C3d,g antibody used in this study also recognizes native C3, we cannot positively exclude the possibility of a passive deposition of native C3 and the existence of a by-pass mechanism for the activation of the terminal pathway (e.g. C5 activation with non-complement enzymes) [24]. Nevertheless, the possibility of a passive deposition of C3 may be definitively ruled out at least in eight patients who had perivascular deposits detected with the polyclonal anti-C3d,g antibody and not with the polyclonal anti-C3 antibody. So, they had only activated C3 perivascular deposits, reflecting involvement of the early pathway of complement activation. Among these eight patients, five also had perivascular deposits of TCC. So, at least for them, it is possible to assume the involvement of the whole cascade of complement, i.e. cleavage of C3 to C3b, activation of the terminal pathway and then formation of TCC.

In an effort to link abnormal complement activation and tissue damage in LCV, TCC has been proposed to be a pathogenic mediator, as it could be found in perivascular deposits in LCV lesional skin [5,11,12]. TCC can be responsible for endothelium destruction either directly, through its cytolytic potential, or indirectly through non-cytolytically induced effects such as the release of arachidonic or oxygen metabolites by endothelial cells [16]. Therefore, there is increased interest in analysing the molecular form of TCC in immune deposits. In the present study we showed that, in LCV skin perivascular immune deposits, clusterin, when demonstrated, was systematically accompanied at the same location by TCC perivascular deposits. Clusterin was not found in the absence of TCC. French *et al.* made the same observation in a recent related immunofluorescence study of lupus band test skin diseases [25]. As suggested by these authors, clusterin may not bind itself to surrounding membrane, but only associated to the TCC. Consequently, this suggests that the TCC in LCV skin immune deposits would be, at least in part, in a non-cytolytical form containing clusterin. Cutaneous endothelial cells are well protected against complement attack by both the presence of membrane-bound regulatory proteins, DAF [13], MCP [14], and CD59 [15] and plasma regulatory proteins factor H, factor I, vitronectin and clusterin. Factor H and factor I were also found to be secreted by endothelial cells [26–28]. Thus, the presence in LCV perivascular deposits of C3d,g and clusterin-associated TCC, which are products of C3b and TCC inactivation respectively, emphasizes the fact that membrane-bound and/or plasma regulatory proteins may adequately fulfil their control role. Nevertheless, there is a possibility that the clusterin-associated TCC form in immune deposits may represent only a part of TCC, accompanied by an active cytolytical form. In this case, the generation of cytolytic TCC may be secondary to a temporary local overload of TCC, due to excessive complement activation.

What can we conclude about the TCC molecular form in LCV immune deposits when TCC perivascular deposits were not accompanied by clusterin perivascular deposits? Soluble TCC, assumed to be inactive, may be found independently with either clusterin or vitronectin [29], but Bhakdi *et al.* [30] showed that cytolytically active TCC contained some vitronectin. So, vitronectin may not be highly specific for a non-cytolytical form of TCC. As suggested by Tschopp *et al.*, clusterin would be the essential soluble inhibitor of TCC [31]. Tending to support this hypothesis, French *et al.* [25] demonstrated that, in lupus band test skin diseases, vitronectin deposits are observed only in the presence of clusterin deposits. In our study, we did not examine vitronectin. So, although clusterin appears to be the best soluble marker for non-cytolytical TCC deposits, we cannot rigorously conclude that, in the absence of clusterin in immune deposits, TCC, when found, was in a cytolytical form.

Another important point which remains to be elucidated is the exact participation of TCC in tissue damage. TCC may be directly responsible for endothelium destruction by the above-described mechanisms. Alternatively, the presence of TCC may simply be a marker of an excessive complement activation going beyond cleavage of C3 and C5, with no direct relevance to tissue damage. Then, the generation of earlier complement activation products such as the anaphylatoxins could be responsible for the destructive inflammatory reaction. In 16% of patients, we found C3d,g perivascular deposits without TCC deposits. In these cases, it is possible that the extent of complement activation was not sufficient to overtake the regulatory proteins and lead to generation of TCC. As these patients had clinical and histological evidence of leukocytoclastic vasculitis lesions, this result also emphasizes the probable participation of the local generation of the anaphylatoxins in LCV.

What is the anomaly leading to excessive local complement activation in LCV? It is generally thought that this occurs through deposition of immune complexes in the vessel walls [2,3]. In this study, we found that 56% of patients had perivascular deposits of immunoglobulin which were always demonstrated with C3d,g and TCC deposits. In addition, we found a positive correlation between the intensity of C3d,g and immunoglobulin perivascular deposits. This result strongly favours an immune complex process in the pathogenesis of LCV. Surprisingly, IgA was the most frequent class of immunoglobulin deposited in the involved dermal vessel walls (48% of the patients). This finding suggests that IgA-containing immune complexes may be significantly implicated in the pathogenesis of LCV. Such an IgA immune complex intervention is well documented in Henoch–Schönlein purpura [11,32], and has also been reported in association with RA [33]. However, in the present study we did not find any association with a specific etiology. As IgA has been recently shown to activate complement via the alternative pathway *in vivo* [34], our results suggest that IgA-mediated activation of complement through the alternative pathway might be of significant pathogenic importance in LCV.

Immunofluorescence studies at the epidermal BMZ revealed moderate or intense deposits of C3d,g in the biopsy specimens from patients with LCV. However, this result does not appear specific for LCV, as in biopsy specimens from control groups 1 and 2 we also constantly observed deposits of C3d,g at the BMZ. These findings are in accord with Basset-Seguin *et al.* [35], who reported the presence of C3d,g in normal human epidermal BMZ. Of interest, we report for the first time the deposition of TCC, in addition to C3d,g, deposits, at the BMZ in biopsy specimens from 52% of patients with LCV. No TCC deposits were found at the BMZ in biopsy specimens from healthy donors or subjects in control group 2. Complement activation at the BMZ in LCV remains difficult to interpret, as no immunoglobulin deposits were found at this level (with the exception of three biopsy specimens from patients with LCV associated with SLE or rheumatoid vasculitis). It is interesting to note that no deposit of clusterin at the BMZ has been observed. As histological analysis of the skin biopsies did not reveal significant alteration of the dermal–epidermal junction, the significance of TCC deposition at the BMZ remains to be determined.

Another important finding of this study are signs of complement activation in plasma from patients with LCV. Levels of C3d,g and TCC in plasma were both increased compared with healthy donors and control group 2 subjects. This was in contrast with the measurement of native complement proteins in plasma, which were poorly able to detect complement activation. Thus in LCV, as in SLE or RA [6–10], C3d,g and TCC appear to be sensitive indicators of systemic complement activation. Measurements of CH_{50} in plasma revealed a significant increase in 65% of the patients. This unexpected result suggests a hypersynthesis of complement acute-phase proteins such as C3 and factor B, in response to their consumption as detected with C3d,g measurements. It is likely that the important variation of C3 and factor B levels within normal ranges does not allow detection of a significant increase of individual C3 or factor B synthesis. Supporting this hypothesis of a reactional hypersynthesis, whenever CH_{50} appeared increased, C3 and factor B plasma levels were found at the upper limit of normal. The lack of correlation between levels of C3d,g and TCC in plasma and the intensity of perivascular cutaneous deposits indicates that the presence of these complement activation products in plasma did not result from a passive diffusion from cutaneous lesion sites to the bloodstream, but rather reflected systemic complement activation. Finally, the good correlation observed between the severity of vasculitis and the elevation of C3d,g and TCC levels in plasma, makes these markers of potential interest in the monitoring of clinical evolution of LCV.

ACKNOWLEDGMENTS

This study was supported by INSERM and the University of Rouen. The authors would like to thank Mrs A. Valauney for technical assistance in immunofluorescence studies, Mr J. Menard for his help in statistical analysis and Mrs A. Chaube for typing this manuscript. H.D. is a fellow of the Ministère de la Recherche et de la Technologie.

REFERENCES

1 Hunder GG, Arend WP, Bloch DA *et al.* The American College of Rheumatology 1990 criteria for the classification of vasculitis. Arthritis Rheum. 1990; **33**:1065–144.

2 Smoller BR, McNutt NS, Contreras F. The natural history of vasculitis. What the histology tells us about pathogenesis? Arch Dermatol 1990; **126**:84–89.

3 Moore PM. Immune mechanisms in the primary and secondary vasculitides. J Neurol Sc 1989; **93**:129–45.

4 Sams WMJr, Claman HN, Kohler PF *et al.* Human necrotizing vasculitis: immunoglobulins and complement in vessel walls of cutaneous lesions and normal skin. J Invest Dermatol 1975; **64**: 441–5.

5 Boom BW, Coby J, Out-Luiting CJ *et al.* Membrane attack complex of complement in leukocytoclastic vasculitis of the skin. Arch Dermatol 1987; **123**:1192–5.

6 Julen N, Dauchel H, Lemercier C *et al.* Measurement of the complement system. General principles and prospects. Ann Biol Clin 1990; **48**:349–60.

7 Morrow WJW, Williams DJP, Ferec C *et al.* The use of C3d as a means of monitoring clinical activity in systemic lupus erythematosus and rheumatoid arthritis. Ann Rheum Dis 1983; **42**:668–71.

8 Kerr LD, Adelsberg BR, Schulman P *et al.* Factor B activation products in patients with systemic lupus erythematosus. A marker of severe disease activity. Arthritis Rheum 1989; **32**:1406–13.

9 Makinde VA, Senaldi G, Jawad ASM *et al.* Reflection of disease activity in rheumatoid arthritis by indices of activation of the classical complement pathway. Ann Rheum Dis 1989; **48**:302–6.

10 Gawryl MS, Chudwin DS, Langlois PF *et al.* The terminal complement complex, C5b9, a marker of disease activity in patients with systemic lupus erythematosus. Arthritis Rheum 1988; **31**: 188–95.

11 Kawana S, Shen GH, Kobayashi Y *et al.* Membrane attack complex of complement in Henoch–Schönlein purpura skin and nephritis. Arch Dermatol Res 1990; **282**:183–7.

12 Boom BW, Mommaas M, Daha MR *et al.* Complement-mediated endothelial cell damage in immune complex vasculitis of the skin: ultrastructural localization of the membrane attack complex. J. Invest Dermatol 1989; **93**:68–72.

13 Sayama K, Shiraishi S, Shirakata Y *et al.* Characterization of decay-accelerating factor (DAF) in human skin. J Invest Dermatol 1991; **96**:61–64.

14 Sayama K, Shiraishi S, Shirakata Y *et al.* Expression and characterization of membrane co-factor protein (MCP) in human skin. J Invest Dermatol 1991; **97**:722–4.

15 Meri S, Waldmann H, Lachmann PJ. Distribution of protectin (CD59), a complement membrane attack inhibitor, in normal human tissues. Lab Invest 1991; **65**:532–7.

16 Morgan BP. Complement membrane attack on nucleated cells: resistance, recovery and non-lethal effects. Biochem J 1989; **264**: 1–14.

17 Murphy B, Kirszbaum L, Walker ID *et al.* SP-40,40, a newly identified normal human serum protein found in the SC5b-9 complex of complement and in the immune deposits in glomerulonephritis. J Clin Invest 1988; **81**:1858–64.

18 Kirszbaum L, Sharpe JA, Murphy B *et al.* Molecular cloning and characterisation of the novel, human complement-associated protein, SP-40,40: a link between the complement and reproductive systems. Embo J 1989; **8**: 711–8.

19 Murphy BF, Davies DJ, Morrow W *et al.* Localization of terminal complement components S-protein and SP-40,40 in renal biopsies. Pathology 1989; **21**:275–8.

20 Fritz IB. What is clusterin? Clin Exp Immunol 1992; **88**:375.

21 Hugo F, Jenne D, Bhakdi S. Monoclonal antibodies against neoantigens of the terminal C5b-9 complex of human complement. Biosci Rep 1985; **5**:649–58.

22 Kazatchkine M, Hauptmann G, Nydegger U. Techniques du complément, INSERM Ed., Paris, 1985:104.

23 Mlynek Y, Nilsson M. The quantitation of C3d by routine methods after the direct absorption of human plasma with anti-C3c. Acta Path Microbiol Immunol Scand Sect C 1985; **93**:195–8.

24 Gay-Crosier F, Polla LL, Tschopp J *et al.* Complement activation by pulsed tunable dye laser in normal skin and hemangioma. J Invest Dermatol 1990; **94**:426–31.

25 French LE, Polla LL, Tschopp J *et al.* Membrane attack complex (MAC) deposits in skin are not always accompanied by S-protein and clusterin. J Invest Dermatol 1992; **98**:758–63.

26 Dauchel H, Julen N, Lemercier C *et al.* Expression of complement alternative pathway proteins by endothelial cells. Differential regulation by interleukin-1 and glucocorticoids. Eur J Immunol 1990; **20**:1669–75.

27 Julen N, Dauchel H, Lemercier C *et al. In vitro* biosynthesis of complement factor I by human endothelial cells. Eur J Immunol 1992; **22**:213–7.

28 Brooimans RA, Hiemstra PS, Van Der Ark A *et al.* Biosynthesis of complement factor H by human umbilical vein endothelial cells. Regulation by T cell growth factor and IFN-gamma. J Immunol 1989; **142**:2024–30.

29 Choi NH, Nakano Y, Tobe T *et al.* Incorporation of SP-40,40 into the soluble membrane attack complex (SMAC, SC5b-9) of complement. Int Immunol 1990; **2**:413–7.

30 Bhakdi S, Kaflein R, Halstensen TS *et al.* Complement S-protein (vitronectin) is associated with cytolytic membrane-bound C5b-9 complexes. Clin Exp Immunol 1988; **74**:459–64.

31 Tschopp J, Shafer S, Jenne DE. Inhibition of the lytic terminal complement pathway by a novel cytolysis inhibitor (CLI), but not by S-protein/vitronectin in plasma (Abstract). Complement Inflam 1989; **6**:406.

32 Baart DE LA Faille-Kuyper EM, Kater L, Kooiker CJ *et al.* IgA-deposits in cutaneous blood vessel walls and mesangium in Henoch–Schönlein syndrome. Lancet 1973; i:892–3.

33 Miyagawa S, Shiiki H, Nakatani C *et al.* Association of IgA immune complex vasculitis and rheumatoid arthritis. J Am Acad Dermatol 1991; **24**:295–7.

34 Stad RK, Bogers WM, Thoomes-Van der Sluys ME *et al. In vivo* activation of complement by IgA in a rat model. Clin Exp Immunol 1992; **87**:138–43.

35 Basset-Seguin N, Dersookian M, Cehrs K *et al.* C3d,g is present in normal human epidermal basement membrane. J Immunol 1988; **141**:1273–80.