Cross – Reactivity of the V3-Specific Antibodies with the Human Clq

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It has been previously shown that the sequence similarity between a portion of the envelope glycoprotein 120 (gp120) from the human immunodeficiency virus type 1 (HIV-1) and several types of human collagen and collagen-like molecules exists. That observation led to the suggestion that the antibodies against the third hypervariable region (V3) of HIV-1 gp120 (V3-specific antibodies) might have a role in the autoimmune phenomena observed in HIV-infected persons. In this study we have examined the cross-reactivity of the V3-specific antibodies purified from sera of HIV-infected individuals, sera obtained from the rheumatoid arthritis and systemic lupus erythematosus patients, as well as from the sera of healthy volunteers with the separate chains of a subcomponent of the first component of the human complement system, Clq. Our results show that the V3-specific antibodies are present in the sera of the HIV-infected individuals, patients suffering of the systemic autoimmune diseases as well as in the sera of healthy volunteers. Whereas these antibodies appeared in the HIV+-sera after antigen challenge, those present in the HIV - sera probably represent the antibodies that are cross-reactive with the antigen. V3-reactive antibodies can be purified by affinity chromatography and they were highly specific for the V3-peptide. Additionally, they showed cross-reactivity with the separate chains of the human Clq as well as with the chicken collagen type VI. Possible physiological implications are discussed.

Introduction

The main binding region for the human immunodeficiency virus type 1 (HIV-1) neutralising antibodies, i.e. the “principal neutralising determinant” (PND), is located within the third hypervariable loop in the V3 region of the envelope glycoprotein, gp120 (Rusche et al., 1988; Palker et al., 1988; Matsushita et al., 1988). Infection by HIV induces a vigorous immune response, especially against its principal neutralising determinant, V3 region. The high titer of IgG and IgA antibodies have been detected at the early stage of the HIV – infection (Kozlowski et al., 1994).

Previously, Metlaš and co-workers (Veljković and Metlaš, 1990; Metlaš et al., 1994a), have reported the sequence similarity between a portion of HIV-1 gp120 envelope protein and human immunoglobulin variable region (IgVh). The similarity has been confirmed by several criteria that have led to the suggestion that gp120 or V3 loop may play a role in autoimmune phenomena, already observed in HIV-infected persons (Pinto et al., 1994; Levy J. A., 1993; Muller et al., 1992; Lake et al., 1994; Grant et al., 1990; Veljković and Metlaš, 1993). In further studies, the sequence homology between a V3 loop of HIV-1\textsubscript{NY5} and several types of human collagen, including the collagenuous tail of human Clq A chain have been reported. Affinity purified V3-reactive antibodies from sera of AIDS patients were reactive with the collagen consensus peptide and with human Clq bound to a microtiter plate (Metlaš et al., 1994b). Based on these studies it has been concluded that this level of sequence similarity can cause the antibody cross-reactivity.

The present study should further examine the cross-reactivity of the V3-specific antibodies with purified human Clq. Sera of the HIV-infected individuals (HIV+-sera), sera of the rheumatoid arthritis (RA) patients, systemic lupus erythematosus (SLE) patients and sera of healthy volunteers (NH-sera) were tested for their reactivity with the peptide synthesised according to the sequence of the third hypervariable region of the HIV-1\textsubscript{NY5} gp 120 envelope protein (V3-peptide) and with...
purified human Clq. The V3-specific antibodies were purified from human sera by affinity chromatography and their reactivity towards purified human Clq was studied as well.

**Materials and Methods**

**Materials**

Peptides and proteins

V3 peptide (K-K-G-I-A-I-G-A-G-R-T-L-Y), synthesised according to the sequence of HIV-1 NYS gp120 third hypervariable region (V3-peptide), then the peptide corresponding to the sequence of the collagenous part of the human Clq A chain (K-K-G-E-A-G-R-P-G-R) and the control peptide (KP) with the sequence unrelated to V3-peptide, were generous gift from Dr. Š. Pongor (ICGEB, Trieste, Italy). The subcomponent of the first component of the human complement, Clq, was purified from pool of sera of healthy volunteers, according to the procedure developed at the Institute for Medical Research, Military Medical Academy, Belgrade (Yugoslavia). Protein concentration in the sample was determined by commercial BCA protein assay kit (Pierce, Rockford, Illinois, USA). The presence and purity of Clq was examined and confirmed by radial immunodiffusion, immunoelectrophoresis and Western blot. Human serum albumin and IgM antibodies were also detected.

**Human sera**

All human sera were separated after blood coagulation by centrifugation at $1500 \times g$ for 15 min. Human immunodeficiency virus was inactivated by heating of HIV+ sera at $56^\circ C$ for 60 min. Also all other sera, i.e. sera obtained from rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) patients and healthy volunteers (NH) were prepared under the same conditions.

**Methods**

Peptide coupling to carrier protein (BSA)

Peptide used in this study was coupled to the carrier protein, BSA, by using 1-ethyl-3-(dimethylammonopropyl) carbodiimide hydrochloride (EDC). Briefly, BSA was dissolved in distilled water (2 mg in 200 μl water), and 2 mg of V3-peptide was dissolved in 500 μl of MES-buffer (0.1 M 2-(N-morpholino) ethanesulfonic acid), 0.9% NaCl, 0.02% NaN₃, pH 4.5). Solutions were mixed and 10 mg of EDC was added. The reaction mixture was left at room temperature for 2 h and then dialysed overnight against 1 mM phosphate buffered saline (PBS). The solution of a coupled peptide were stored at $-20^\circ C$ until use.

Enzyme linked immunosorbent assay (ELISA)

ELISA assays were performed in order to determine the sera and affinity purified antibody reactivity with V3-peptide. The antigen – V3-BSA, Clq-BSA or KP-BSA – was bound to 96-well polystyrene microtiter plate by incubation for 2 h at $37^\circ C$ in 0.1 M carbonate buffer pH 9.5. After rinsing with 0.1 M phosphate buffered saline (PBS) / 0.5% Tween 20, pH 7.4 buffer, microplates were saturated with 1% BSA solution in PBS for 1 h at room temperature. The plates were rinsed three times and then the primary antibodies were added. RA, SLE, and NH sera were incubated with V3-BSA diluted 100-fold, whereas the HIV+ sera were diluted 1000 fold with PBS. Anti-human Clq antiserum was incubated with V3-BSA in various dilutions (1:500, 1:1000, 1:1500, 1:2000, and 1:2500). After purification by affinity chromatography (described below) the V3-specific antibodies were incubated with V3-BSA and KP-BSA in amount of 0.0625 μg/well. Primary antibodies were incubated for 1h at room temperature, and after rinsing with PBS/0.5% Tween 20, pH 7.4 buffer, secondary horseradish peroxidase (HRP) conjugated anti-IgG or anti-IgM antibody was added. This 1h-incubation step was followed by rinsing with PBS. The colour was developed by adding ABTS substrate (0.05% 2,2'-azino-di(3-ethyl)-benzethiazoline sulfonic acid (ABTS), 0.03% H₂O₂, 0.1 M citrate buffer, pH 4). After 15 min, the reaction was terminated by 0.1 M citrate buffer, pH 1.8, and the absorbance at 405 nm (A₄₀₅) was measured. All dilutions were made in 0.1 M PBS 0.1% BSA, pH 7.4. Each sample was measured in duplicate, and the mean values of the blank probe were subtracted from each individual values. Only those samples showing an A₄₀₅ twice higher than the corresponding blank were considered reactive towards the antigen.
Affinity purification of antibodies

V3-reactive antibodies were affinity purified from HIV+, RA, SLE, and NH sera on the V3-BSA Sepharose prepared according to the manufacturer's instruction. Sera were dialysed overnight against PBS, and then incubated with V3-BSA Sepharose for 2 h at 4 °C. After rinsing the resin with PBS, bound antibodies were eluted with 0.1 m acetate buffer, pH 3, and fractions were immediately neutralised with 2 m Tris (tris-(hydroxy-methyl)-aminomethane), pH 9. Elution was monitored by measuring the absorbance at 280 nm (A280), and fractions with A280 values higher 70% of maximal value were collected for further analyses. Pooled fractions were analysed by SDS-PAGE. Only in sample of antibodies purified from RA sera presence of another protein beside antibodies was detected.

SDS-PAGE and Western blot

In order to examine the sera and affinity-purified antibody reactivity with separate chains of human Clq, sample of purified Clq was resolved on 10% polyacrylamide gel under non-reduced and reduced conditions according to Laemmli (Laemmli, 1970), and then transferred onto the nitro-cellulose membrane as described in (Towbin et al., 1979). Briefly, purified Clq was mixed in 1:1 (v : v) ratio with the non-reducing sample buffer (0.2 m Tris, 4% sodium dodecylsulfate (SDS), 20% glycerol, 0.02% bromphenol blue) or with the reducing buffer (0.2 m Tris, 4% sodium dodecylsulfate (SDS) 20% glycerol, 0.02% bromphenol blue, 6% β-mercaptoethanol) and left overnight prior to application on gel. After separation, proteins were transferred from gel to nitro-cellulose membrane. After blocking of the membrane with 1% bovine serum albumin for 3 h at room temperature, nitro-cellulose membranes were incubated with primary antibodies. Mouse anti-human Clq antiserum was incubated with purified Clq samples separated under non-reduced (sample buffer did not contain β-mercaptoethanol) and reducing (sample buffer contained β-mercaptoethanol) conditions, as well as human sera diluted with tris buffered salt solution (0.02 m Tris 0.5 m NaCl, pH 7.5) with 0.05% Tween 20 (TTBS). Also, affinity-purified antibodies from HIV+, RA, SLE and NH sera were tested for their reactivity with both forms of the sample of human Clq. Incubation of the sample of Clq with primary antibodies was 1h at room temperature. After washing with TTBS secondary anti-IgG and anti-IgM antibodies were incubated with nitro-cellulose sheets, and protein bands were detected with 3,3'-diaminobenzidine (DAB) as a substrate (0.03% DAB, 10 mm phosphate buffer, pH 6.5, 0.01% H2O2). Each incubation step was followed by rinsing nitro-cellulose with TTBS (3 times, 5 min each).

Statistical analysis

Comparison of the sera reactivity with the peptides was done by Student's t-test and if p < 0.05 the difference was considered significant.

Results

Reactivity of human sera with the V3-peptide

In the first part of our work we have tested human sera for their reactivity with the V3-peptide coupled to the carrier protein, BSA. Fig. 1 represents the mean reactivity of the tested sera with the V3-peptide coupled to BSA (V3-BSA). The HIV +, RA-, SLE- and NH-sera reactivity with

![Fig. 1. Reactivity of human sera with V3-peptide. Sera of healthy volunteers (NH), patients suffering of rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) as well as of the sera obtained from HIV-infected patients (HIV +) were incubated with V3-peptide coupled to BSA (V3-BSA). The HIV +, RA-, SLE- and NH-sera reactivity with](image)
the V3-peptide was examined by ELISA, and the reactivity of IgG and IgM antibodies was detected. All sera showed the reactivity of IgG and IgM antibodies towards the V3-peptide. The mean value obtained for IgG antibodies of HIV+-sera (0.88 ± 0.35) was higher than that obtained for other sera (0.25 ± 0.07, 0.22 ± 0.07, and 0.16 ± 0.09 for RA, SLE and NH sera, respectively). The mean IgG antibody reactivity of RA- and SLE-sera was not significantly higher in comparison to that measured for NH-sera (p < 0.05).

The mean value for IgM antibodies of the RA-sera (0.86 ± 0.10) was highest of the IgM antibody reactivity among other human sera tested (0.54 ± 0.3, 0.73 ± 0.10, and 0.37 ± 0.20, for HIV+-, SLE- and NH-sera, respectively). Rather high standard deviation is obtained for HIV+ sera, especially in the case of IgG antibodies. Only few of the HIV+- sera tested showed lower level of the V3-specific reactivity then NH-sera.

The reactivity of mouse anti-Clq antiserum with the V3-peptide

In order to obtain more information about the proposed antigenic similarity between two unrelated proteins – gpl20 from HIV-1_NY5 and human C1q – mouse antiserum raised against human C1q was tested at various dilutions for its reactivity with V3-BSA. Fig. 2 shows the reactivity of the IgG antibodies of the mouse anti-Clq antiserum

with the V3-peptide tested by ELISA. The reactivity of the anti-C1q antiserum with the V3-peptide decreased with increasing dilution, and the shape of the dilution curve indicates a rather low concentration of IgG V3-reactive antibodies in the antiserum. However, these data indicate that a certain degree of cross-reactivity of the anti-human C1q antiserum with the V3-peptide exists.

The reactivity of the human sera and the affinity-purified antibodies with V3- and Clq-peptide

In the next step of our experiments we have purified the V3-reactive antibodies from human sera by affinity chromatography as described in the “Materials and Methods” section. Fig. 3a shows the yield of the V3-specific antibodies from HIV+-, RA-, SLE- and NH-sera calculated per volume of the applied serum. The highest yield of proteins was obtained from HIV+-sera, whereas the lowest amount of proteins was purified by affinity chromatography from NH-sera. Purified antibodies were tested for their reactivity against V3-peptide by ELISA. The results presented in Fig. 3b show that both IgG and IgM antibodies were purified from sera. By comparison the reactivity of an individual antibody class, the results are in accordance with those obtained with sera (cf. Fig. 1): The IgG antibodies from HIV+-sera were of the highest reactivity (all affinity-purified antibodies were applied at the same concentration). The IgM antibodies of the RA-sera were the most reactive with V3-peptide among other IgM antibodies.

In order to check the specificity of the affinity-purified antibodies, all samples were tested for their reactivity towards the unrelated peptide, control peptide (KP-BSA). In Fig. 3c, the reactivity of the V3-specific antibodies purified from human sera towards KP-BSA is presented. The control peptide used in our study has the same amino acid composition as V3-peptide but with different sequence, and was used as a control for the antibody specificity. All affinity-purified antibodies showed low reactivity with KP-BSA. The highest reactivity was detected in the sample purified from RA-sera (IgM antibodies) and it does not exceed 20% of the same antibody reactivity with V3-peptide (Fig. 3b).

HIV+- and NH-sera as well as the affinity-purified V3-specific antibodies from HIV+-and NH-
Fig. 3. Yield of V3-specific antibodies affinity purified from human sera (a) and their reactivities with V3- (b) and KP- (c) peptides. V3-specific antibodies were purified on V3-BSA-Sepharose. Chromatography was monitored by absorbance at 280, and the protein concentration in the eluate was per ml of a serum. Reactivity of such a purified antibodies with V3- and KP- peptides was tested by ELISA. Equal amounts of proteins were incubated with microtiter-plate coated peptides, and the reactivity of IgG and IgM antibodies was detected by HRP-conjugated secondary antibodies. The data represent the mean of two independent measurements.

NH and HIV+ sera showed weak reactivity with the peptide (data not shown). In accordance to the results obtained with the V3-specific antibody reactivity against the Clq-peptide, reactivity of the affinity purified V3-specific antibodies was not detected with the peptide synthesised according to the sequence homologous to V3 of the human collagen type II (data not shown).

The reactivity of human sera and the affinity-purified antibodies with human Clq

The reactivity of HIV+, RA, SLE- and NH- sera with human Clq was examined by Western blotting. Human Clq was purified from a pool of sera of healthy volunteers. The human serum albumin and IgM antibody were also detected (data not shown). The presence of human Clq was confirmed by positive reaction with anti-human Clq antiserum by immunoelectrophoresis, immunodiffusion and Western blotting.

The sera reactivity with the purified Clq separated electrophoretically under non-reducing and reducing conditions was tested. None of the sera examined showed neither IgG nor IgM antibody reactivity with non-reduced Clq (data not shown). Also, the HIV+ and NH- sera did not react with the reduced sample of human Clq under our ex-
Fig. 5. The reactivity of human sera (a) and the V3-specific affinity purified antibodies (b) with purified Clq separated electrophoretically under reducing conditions (sample buffer containing β-mercaptoethanol). The reactivity was tested by Western blot with Clq bound to the nitro-cellulose membrane. The binding of the antibodies was detected by the anti-IgG (presented in the figure) and anti-IgM secondary, HRP-conjugated antibodies, and 3,3’-diaminobenzidine (DAB) as substrate. The results are representative of three independent samples. Position of three individual chains of human Clq – A, B and C – is indicated in the figure.

Experimental conditions (Fig. 5a), whereas RA- and SLE- sera appeared to be reactive with the separate chains of the reduced human Clq (Fig. 5a). Additionally, RA- sera showed primarily the reactivity of IgM antibody, whereas SLE- sera showed both IgG and IgM antibody reactivity against reduced sample of purified human Clq.

Accordingly, the reactivity of the affinity-purified, V3-specific antibodies, against purified Clq that was electrophoretically separated under non-reducing conditions was not detected (data not shown). On the other hand, IgG and IgM V3-specific antibodies from all human sera – HIV+, RA, SLE and NH – were reactive with A- and B-chains of the reduced Clq (Fig. 5b). The individual chains of human Clq – A, B and C – were recognised according to their molecular masses.

In separate experiments, human sera as well as the affinity purified V3-specific antibodies were tested for their reactivity with the chicken collagen type VI by ELISA. The protein was bound to the microtiter plate, and the reactivity of both IgG and IgM antibodies was detected. Whereas all sera failed to react with the chicken collagen type VI (data not shown), the affinity-purified, V3-specific antibodies showed slight reactivity of IgG and IgM antibodies (data not shown). However, their reactivity was between 5-fold (NH sera) and 15-fold (HIV+-sera) lower than with the V3-peptide as assessed by the same assay.

Discussion

Antigenic similarity between A chain of human Clq and V3 region of gp120 was assumed on the basis of high degree of sequence similarity that has been found by Metlaš and co-workers (Metlaš et al., 1994a). Lack of the cross-reactivity between V3-specific antibodies affinity purified from HIV+, and NH-sera, towards the Clq-peptide – synthesised according to the homologous region of Clq A chain – probably is caused by the absence of some amino acids that might be important for the epitope formation. This assumption was made by the shown cross-reactivity of the V3-specific antibodies with the A chain of human Clq. Additionally, the epitope(s) similar to V3 region of gp120 HIV-1_NYS on human Clq B chain was detected (Fig. 5b).

Peptide synthesis and examination of antibody cross-reactivity is a common approach for locating the antigenic determinant or epitope of a particular protein. Any linear peptide of 5–10 amino acids that is found to react with antibodies is considered a »continuous« epitope. Such an epitope within the protein is formed from the neighbouring amino acids. On the other hand, the epitopes might be formed from a rather distant amino acids, and those are called »discontinuous«. In some cases, the epitope conformation presented by a peptide, might be important for antibody reactivity (Van Regenmortel, 1987).

High level of the anti-V3 antibodies in HIV-infected sera was detected in our experiments (Fig. 1), and the highest amount of V3-rective antibodies was purified from the HIV+-sera compared to RA, SLE and NH sera (Fig. 3a). This again indicates that although a high degree of the sequence similarity between HIV-epitopes and some human proteins exists (Metlaš et al., 1994a) the immune system recognises viral epitopes as foreign determinants. The immune response initiated against the determinants similar to the V3 might cross-react with closely homologous self-host proteins such as collagens or Clq. Also, the polyclonal mouse anti-human Clq antiserum binds to the V3 peptide (Fig. 2), and therefore, the cross-reactivity between these molecules with the sequence similarity occurs.
Probably due to the antigenic similarity between host proteins and viral epitope(s), we detected antibodies reactive with V3 loop-derived peptide, which was not initially present in sera of patients with rheumatoid arthritis and systemic lupus erythematosus (Fig. 1). The antibodies were mainly IgM class, while IgG antibodies showed no marked differences compared to control sera, which again suggest the polyclonal activation of B lymphocytes in this systemic autoimmune disorders (Jeffersis, 1995). We suppose that these are antibodies raised against C1q or collagen that are often detected in sera of patients with systemic autoimmune diseases (Wisnieski and Jones, 1992; Rönneled and Klarekskog, 1995; D’Cruz et al., 1995; Coremans et al., 1995; Wisnieski and Naff, 1989; Wisnieski and Jones, 1992; Siegert et al., 1990). These antibodies could cross-react with similar epitopes. Anti-collagen and anti-C1q autoantibodies are directed against those molecules that are conformationally changed or denatured. Only in a few cases autoantibodies directed against native collagen molecules has been detected. (Clague and Moore, 1984; Andriopoulos et al., 1976).

We detected neither human sera nor affinity purified antibody reactivity with the non-reduced human C1q, which is in accordance with these previous reports. In our experiments, the sera failed to react with the chicken collagen type VI bound to the microtiter plate, most probably due to the low number of epitopes exposed in this non-denatured proteins. However, the low but detectable cross-reactivity of the V3-reactive antibodies purified from human sera with the same protein, indicates that the V3-similar epitopes also exist on the chicken collagen type VI.

Results of numerous authors suggest that autoantibody production against these determinants is a secondary event compared to the inflammatory reactions. Presence of the enzymes and reactive oxygen species released during immune reaction against infection might damage C1q, which is also involved in defence mechanism. Changes in conformation C1q might expose its cryptic epitopes to the immune system. In addition, it has been shown that after limited proteolysis of C1q by leukocyte collagenase this protein is antigenically similar to collagen type II (Heinz et al., 1988; Heinz et al., 1989). We detected the reactivity of the affinity purified antibodies only with a reduced sample of C1q (Fig. 5b), which is in accordance with those findings. Conformational changes induced when unreduced C1q was bound to the nitro-cellulose membrane probably were not sufficient to detect the antibody binding. Additionally, reduction of the antigen leads to detectable antigenic cross-reactivity of the V3 reactive antibodies from HIV+-, RA-, SLE- and NH- sera.

IgM anti-gp120 antibodies were detected in sera of healthy individuals, and it appears that they are a part of normal immune repertoire (Pinto et al., 1994). In our studies we detected IgM V3 reactive antibodies in sera of healthy volunteers (Fig. 1). Very low IgG reactivity in control sera was found. Very recently, the presence of the V3-reactive IgG antibodies in normal human sera was described (Metlaš et al., 1999a; Metlaš et al., 1999b). In sera of HIV-infected individuals numerous autoreactive antibodies are detected and it has been suggested that autoimmune phenomena may participate in disease progression. Autoantibodies found in sera of HIV-infected persons are suggested to be triggered by viral antigens with similar antigenic properties with self-host proteins (DeBari and Akl, 1994; Gotoh and Matsuda, 1995; Lake et al., 1994; Müller et al., 1994; Theofilopoulos, 1995).

This is one more report about unrelated proteins whose antigenic homology, proposed on the basis of their sequence homology is experimentally shown. Further studies must be performed considering the structural motifs on human C1q B chain which are responsible for antigenic similarity with the third hypervariable region of HIV-1NY5 gp 120.

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