Epstein-Barr Virus–induced Autoimmune Responses

I. Immunoglobulin M Autoantibodies to Proteins Mimicking and Not Mimicking Epstein-Barr Virus Nuclear Antigen-1

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Abstract

In previous studies of infectious mononucleosis, we found IgM autoantibodies which react with hematopoietic cell antigens. Many of these were inhibited by synthetic glycine/alanine peptides representing the glycine/alanine repeat of Epstein-Barr virus nuclear antigen-1. We have cloned and expressed fragments of genes encoding two of these autoantigens. One gene (p542) encodes a protein containing a glycine-rich 28-mer, which is its chief autoantigenic epitope and which represents a newly identified class of evolutionarily conserved autoepitopes. The other gene (p554) encodes a protein that is not demonstrably cross-reactive with Epstein-Barr virus nuclear antigen-1 or with any other EBV protein, but forms complexes with other proteins. Immunoaffinity-purified anti-p542 and anti-p554 have relatively high binding affinities, as evidenced by inhibition at 10^6–10^8 M^{-1}, and neither autoantibody showed polyreactivity with other common antigens. The data thus suggest that neither autoantibody is simply an expression of polyclonal B cell activation. We conclude that the two autoantigens stimulate autoantibody synthesis by different mechanisms. One autoantigen shares homology to a viral protein which generates cross-reacting antibodies to the autoantigenic epitope. The other has no recognizable cross-reaction with the infecting pathogen and may become immunogenic through complexing with other proteins. (*J. Clin. Invest.* 1995. 95:1306–1315.) Key words: glycine • competitive binding • B lymphocyte • recombinant fusion protein • autoepitope

Introduction

Infectious mononucleosis (IM)[1] is characterized by the generation of a variety of autoantibodies (1–9). An unusual opportunity exists in IM, therefore, to observe the induction and subsequent evolution of virus-induced autoimmunity, as one follows individuals from preinfection through recovery. We do this here and in reference 10, in part cross-sectionally and in part by serial analyses.

We previously reported (11) that during acute infectious mononucleosis patients produce IgM antibodies not only to EBV-encoded antigens, including Epstein-Barr virus nuclear antigen-1 (EBNA-1) but also to nine or more autoantigens detected as bands in Western blots of myelopoietic cell lines. Antibody eluted from most of the bands, including that from the EBNA-1 band in infected B lymphocytes, reacted with most of the other bands, and they were inhibited by a glycine/alanine (gly/ala) synthetic peptide representing the major antigenic epitope in EBNA-1. The autoantibodies were therefore considered to be examples of autoimmunity by molecular mimicry, i.e., anti-EBNA-1 antibodies that cross-react with autoantigens. During recovery, when the immune response to the EBNA-1 gly/ala repeat switched to IgG, the cross-reactivity with the autoantigens was not retained. Thus it seems that, during maturation of the anti-gly/ala part of the anti-EBNA-1 response, IgG antibodies reactive with configurations common to the autoantigens are negatively selected.

We have now cloned the genes encoding five cellular antigens recognized by autoantibodies in IM and have expressed recombinant products of two of them, p542 and p554. The expression product p542 contains a glycine/serine 28-mer, GGGASGGGGGGGGSGGGGSGGGGGGSS, which we show here constitutes its mimicking epitope and its major autoantigenic site. Immunoaffinity-purified IgM anti-p542 reacts with multiple B cell autoantigens, and these reactions are inhibited by synthetic gly/ala and glycine/serine peptides. Anti-p542 also reacts directly with recombinant EBNA-1, as well as with synthetic gly/ala peptides representative of EBNA-1. By these criteria, IgM anti-p542 is an expression of the cross-reactive autoimmunity we previously described (11), with the glycine-rich 28-mer being the basis for the molecular mimicry. Sequences highly homologous to the 28-mer are frequent in proteins listed in the University of Wisconsin database and represent, collectively, a new class of autoepitopes.

Immunoaffinity-purified IgM autoantibody to p554, the other expression product, has no reactivity with EBNA-1 or with gly/ala peptides, nor with any discernible viral antigen in EBV-producing cells. During or after its bacterial production, p554 can form complexes with a wide variety of *Escherichia coli* proteins. This suggests the possibility that the autoantigenicity of p554 in IM may be based on similar complexing, but with viral or autologous proteins.

Methods

Cells. The WiL2 line of B lymphocytes (American Type Culture Collection Rockville, MD) used here is a nonsecreting mutant derivative of

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1. *Abbreviations used in this paper:* CMV, cytomegalovirus; EBNA-1, Epstein-Barr virus nuclear antigen-1; gly/ala, glycine/alanine; IM, infectious mononucleosis; PM, powdered milk.

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an EBV-positive Burkitt's lymphoma, which is deficient in immunoglobulin expression. BJAB is an EBV-negative Burkitt's lymphoma B lymphocyte line (12). The GM 02504G fibroblasts were from the NIGMS Human Genetics Mutant Cell Repository (Bethesda, MD). The K562 erythroleukemic cell line, U937 monocyte line, Raji B lymphocyte line, CEM T lymphocyte line, and HeLa epidermoid carcinoma cell line were all from ATCC. The thymocytes were fresh cells obtained as discarded by-product of medically indicated cardiac surgery (courtesy of Dr. Constantine Tsoukas, San Diego State University). The chondrocytes and synoviocytes were two to three times passaged fresh cell cultures from joint replacement surgery (courtesy of Dr. Martin Lotz, University of California, San Diego).

Sera. Sera were acquired from patients with acute infectious mononucleosis or acute streptococcal sore throat at their first visits to the San Diego State University Student Health Center. Serum SD7 had a high titer of IgM autoantibody activity and was used as the standard probe for B cell autoantigens. The sera of 16–17 yr-old VCA-positive and VCA-negative individuals were from student volunteers at La Jolla High School, La Jolla, CA. The sera from patients with Waldenstrom's macroglobulinemia were the gift of Dr. Hans Spiegelberg, University of California, San Diego.

Recombinant autoantigen preparations. The cloned fragments of p542 and p554 (Rhodes, G. H., J. R. Valbracht, and J. H. Vaughan, manuscript in preparation) were inserted into the pSEM3 (13) and pRSET (Invitrogen, Inc., San Diego CA) expression vectors to create β-galactosidase and hexahistidine fusion proteins, respectively. Both JM109 (Stratagene Inc., La Jolla, CA) and DH5α (GIBCO BRL, Life Technologies, Inc., Gaithersburg, MD) strains of *E. coli* were transformed with pSEM3. The DH5a cells, which produce the recombinant products constitutively, were grown overnight, the cells harvested by centrifugation, washed with PBS, pH 7.3, and lysed by addition of 2 ml of Laemmli sample buffer (14) for each 200 ml of cell culture. The JM109 bacteria (a lac^q strain) produce little recombinant protein until induced with isopropylthiogalactoside. The JM109 were grown overnight at 37°C, and a 1:100 dilution of this grown an additional 4 h to achieve an OD_{550} 0.3–0.6, after which the cells were induced with isopropylthiogalactoside in a second overnight incubation. Crude preparations of the β-galactosidase fusion proteins (called p542-B and p554-B) were prepared from the bacteria by centrifugation at 3,000 *g*, dissolution in Laemmli buffer at $8–16 \times 10^9$/ml, heating to 100°C for 4 min, and storage in the frozen state until needed.

Gel purification of the recombinant antigens was achieved by electroelution from thick gel slabs. 2 ml of crude bacterial lysates in Laemmli buffer, or eluates from the nickel columns (see above) were applied to 13 × 17 cm double thickness acrylamide gels. At the end of the run the gel, still affixed to one plate, was submerged in 0–4°C 0.25 M KCl for 5–10 min, after which the recombinant proteins can be seen as distinct white bands (15). These are then sliced out with a scalpel, placed in dialysis tubing, and electroeluted for 1 h into Tris-glycine transfer buffer containing 0.5% SDS and 1 mM PMSF. At the end of the 1 h, the transfer buffer is changed and electroelution continued for a second hour. The two eluates are pooled.

For hexahistidine fusion products, JM109 cells were transformed by the pRSET expression vector (Invitrogen, Inc.) carrying the p542 or p554 inserts. Protein expression was effected by infecting the transformed cells with an M13 phage containing a T7 polymerase gene. The induced bacteria were lysed by sonication in 6 M guanidium buffer and the recombinant protein (called p542-H and p554-H) isolated by adsorption to a nickel column and elution at pH 4–5, as recommended by the manufacturer. Some of these isolates were further purified from gels as described above and designated p542-HG or p554-HG.

Western blots. Bacterial extracts or purified recombinant proteins in Laemmli buffer were electrophoresed in 7.5, 10, or 15% acrylamide gel for 2 h, transferred to nitrocellulose, and then cut into strips and blocked with powdered milk (pm). Autoantibody eluates used to probe the strips (see below) were generally examined undiluted and were allowed to react with the strips overnight before developing with enzyme-labeled anti-Ig. Patients' sera were used as probes at 1/100 dilution and were reacted with the strips for 1–2 h at room temperature overnight at 4°C, before washing and reacting them with labeled anti-Ig.

Extracts of human tissue culture cells were prepared by boiling $0.5–1.0 \times 10^7$ cells for 3–5 min in 1.0 ml of Laemmli buffer and examined by Western blot as above.

Immunoaffinity purification of autoantibody. Bacterial extracts containing the recombinant proteins were electrophoresed in polyacrylamide gels and the proteins electrotransferred to nitrocellulose strips. The levels of the strips containing the autoantigens were cut out, washed, and reacted with autoantibody-containing sera. Antibody was eluted from them at pH 11.5 and then promptly neutralized in PM, essentially as described previously (11). Equal sized strips cut out from other regions of the nitrocellulose were similarly treated, and used as controls.

ELISA. Assays for autoantibodies were carried out in 96-well microtiter trays (Costar Corp., Cambridge, MA), essentially as previously described (16). In brief, the wells were coated with antigen for 1 h at 37°C, the wells blocked with 1% BSA, and the antibody in PM applied for 1 h at room temperature. To assure against artifact from possibly contaminating *E. coli* protein, sera being examined for anti-p542-H were blocked also with extracts of *E. coli* infected by phage-carrying antisense inserts. For anti-p554, it was deemed necessary (see Fig. 2 *A*) to subject p554-H to an additional purification procedure. The nickel column eluates of p554 were further purified by gel, and the 19-kD protein electroeluted into 0.3% SDS. These eluates were free of bacterial antigen, as assessed by a commercial anti-*E. coli* antiserum (Dakopatts, Copenhagen, Denmark). In the final assay, serum antibody activity to the 19-kD p554 was read against a control consisting of the 19-kD zone of bacterial lysate not expressing the recombinant. The OD in this control was always very low or zero.

The detecting antibody was rabbit anti-human IgM or IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) conjugated with horseradish peroxidase. Each antigen was independently evaluated in preliminary studies for optimal concentration for effective coating of the plates, which usually was 10 μg/ml. Sera were examined preliminarily in multiple dilutions and a single dilution, usually 1:100, that was known to be beyond the high dose plateau for the antibody, was selected for most surveys.

Antibody inhibition. Inhibition of antibody staining of Western blots was carried out by incubating the antibody solution with soluble or solid phase synthetic peptide (see below) in PM buffer overnight at 4°C before reaction with the filter. Inhibition of ELISA by soluble keratin or by the 19-kD fragment of p554 was carried out by preincubating the mixtures for 1 h at 37°C before adding them to ELISA plates precoated with antigen at 1 μg/ml.

Peptides. The peptides used in this study (Table I) were synthesized by the solid phase method of Merrifield (17) modified as described (18). To obtain solid phase peptide reactants, aliquots of the peptide solutions were added to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) affinity support beads, according to manufacturer's instructions as follows. 2 ml of peptide solution (1 mg/ml) were combined with 2 ml of washed beads and mixed end-over-end for 3 h at 4°C. Remaining active sites were blocked with ethanolamine. Beads were washed with acid and then basic buffers to remove adsorbed ligand. Validation of the conjugation was obtained in each instance by hydrolysis of the sample and analysis according to Spackman et al. (19) by the Protein Sequencing/Amino Acid Analysis Laboratory of The Scripps Research Institute La Jolla, CA.

Antigens. Keratin was from human epidermis (Calbiochem-Novabiochem Corp., La Jolla, CA). Type II human collagen was the gift of Dr. Steffen Gay, University of Alabama Medical School. Actin, insulin, and bovine thyroglobulin were purchased from Sigma Chemical Co., St. Louis, MO. Pneumococcal vaccine (Pneumovax) and tetanus toxoid were the licensed immunization products of Merck, Sharp and Dohme, Rahway, NJ. The heat shock protein groEL was a recombinant product and the gift of Dr. David Yu, University of California, Los Angeles. The human heat shock protein huHsp60-H is the hexahistidine fusion protein of huHsp60 prepared in this laboratory, as described separately, from the huHsp60 (P1) gene provided to us by Dr. Radhey Gupta, McMaster University, Hamilton, Ontario.

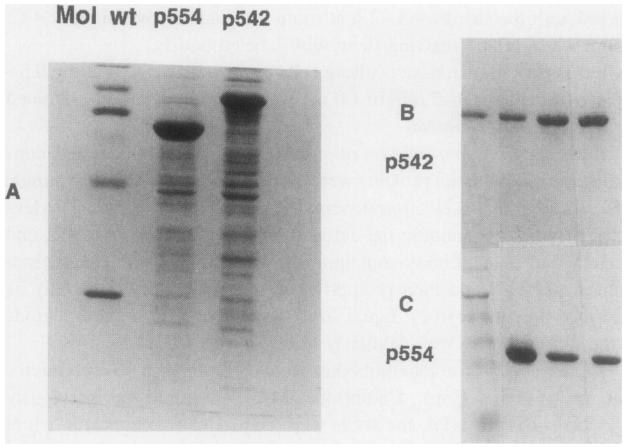

Figure 1. (A) Polyacrylamide gel electrophoresis of lysates of DH5a and JM109 bacteria expressing p554 and p542, respectively, as β-galactosidase fusion proteins. The mol wt markers on the left are at 95, 77, 66, 43, and 30 kD. (*B*) Gel-purified p542 rerun in PAGE in duplicate at 50 (*left* lanes) and 100 μg/ml (*right lanes*). (*C*) Gel-purified p554 rerun in PAGE at 500 (*second lane*) and 100 μg/ml (*right lanes*). (Lane *1*) mol wt markers at 116, 95, 77, 66, 50, and 43 kD.

Induction of cells with PMA. Cells were grown to 10^6/ml, split to 0.5×10^6/ml, and cultured for 3 d in 3 mM sodium butyrate and 20 ng/ml of PMA. They were then washed, sedimented, and boiled in Laemmli buffer at $0.5–1.0 \times 10^7$ cells/ml preparatory to Western blotting. Validation of the activation was made by demonstrating a major increment in the restricted or diffused early antigens using the monoclonal antibodies 85K or R3 (Pearson & Co., Cologne, Germany).

Transfection of COS-7 cells with the EBNA-1 gene. Recombinant EBNA-1 was expressed in COS-7 monkey kidney cells (ATCC). Cells (2×10^6) were plated in 100-mm petri dishes and transfected the next day with 10 μg of plasmid DNA using a cationic lipid formulation based on the lipid DNRIE (20). Cells were washed with PBS 48 h later and lysed by adding 2 ml of Laemmli sample buffer to the plates. The solution was removed to tubes, sheared by passage through a 26-gauge syringe needle, heated for 4 min at 100°C, and stored at −20°C until needed.

The EBNA-1 expression vector was kindly provided to us by Dr. Elliott Kieff (Harvard Medical School, Cambridge, MA). The plasmid has an SV40 early promoter and contains a Sau3AI to PvuII fragment from EBV (map coordinates 107,930–110,177). The DNA was purified using Maxiprep columns (Promega Corp., Madison, WI).

Results

Recombinant β-galactosidase and hexahistidine fusion products. The p542 (~ 1020 bp) and p554 (~ 920 bp) gene fragments were isolated from a λgt11 cDNA Raji cell library; this is reported elsewhere (Rhodes, G. H., J. R. Valbracht, and J. H. Vaughan, manuscript in preparation). As β-galactosidase fusion proteins, p542 and p554 migrated at 71 and 57 kD, respectively, (Fig. 1) and were seen as single bands after isolation from the gels. When probed with a 1:1,000 dilution of the prototype serum SD7 and developed with an anti-IgM reagent, only the 71- and 57-kD bands reacted. The products were designated p542-B and p554-B to specify both the gene product and the fusion partner, respectively.

The hexahistidine fusion protein, p542-H, was isolated from the bacterial lysate as a single band at 30 kD (Fig. 2 *A*). The same procedure used for p554 yielded, in addition to the main 19-kD product, a ladder of lesser bands ranging from ~ 30–~ 100 kD (Fig. 2 *B*). The bands in the ladder reacted with both anti-*E. coli* and anti-p554-B reagents (not shown), indicating that the bands represented p554/*E. coli* protein complexes. Neither p554-B nor p542-H formed such complexes, so the complexes were peculiar to p554-H. We presume that the large β-galactosidase fusion partner of p554-B occluded a p554 binding site for these proteins, a site that was left open in p554-H.

Immunoaffinity purified IgM anti-p542-B and anti-p554-B from serum SD7. Immunoaffinity purified IgM anti-p542-B and anti-p554-B were prepared from the prototype serum SD7 and used as probes in Western blots of WiL2 B lymphoblastoid cells, a cell line latently infected with EBV. The anti-p542-B stained four predominant proteins at 104, 77, 71, and 60 kD (Fig. 3, *left*, lane *1*). Several additional fainter bands not evident in the figure were also seen in the original. The anti-p554-B stained two bands, one a B-cell protein at 72 kD and the other a protein that ran with the dye front (Fig. 3, *right*, lane *1*). In 15% gels, this dye front protein appeared variously as a 26 kD singlet or a 26–28 kD doublet.

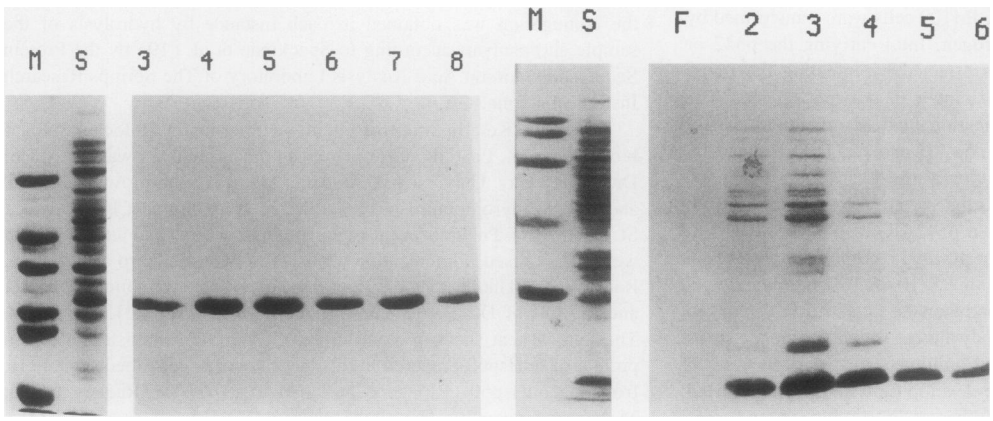

Figure 2. Purification of p542-H (*A*) and p554-H (*B*) fusion proteins by adsorption to nickel columns, elution at pH 4.0, reapplication to the columns, and elution in 300 mM imidazole. Shown are amido black stains of polyacrylamide gel-electrophoresed samples of the original lysates of the bacteria containing the recombinants (lanes *S*), of the last washes of the columns before elution in imidazole (lanes *F*), and of successive (numbered) fractions thereafter of eluates containing the recombinants. All bands in the ladders in (B) were also stained positively both with anti-p554-B and with a rabbit antibody to *E. coli*. Mol wt markers (*M*): 66, 45, 36, 29, 24, and (*A*, lane *4* only) 20 kD.

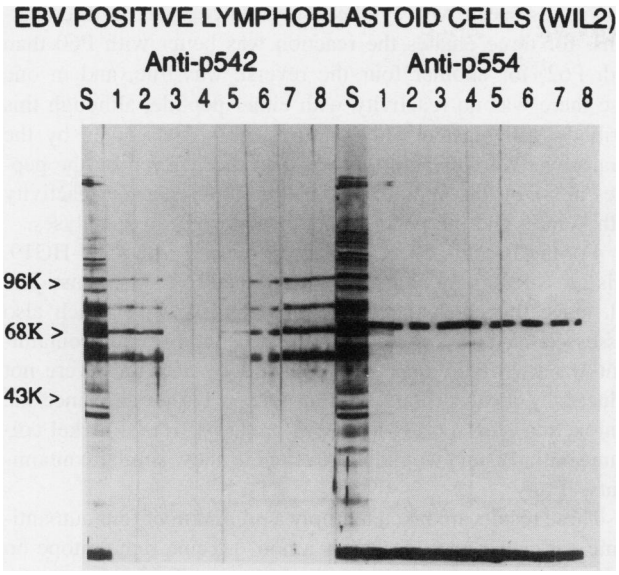

Figure 3. Reactivities and specificities of anti-p542-B and anti-p554-B autoantibodies from serum SD7 in Western blots with whole cell extracts of an EBV-positive B lymphoblastoid cell line (WiL2). The autoantibodies were immunoaffinity purified from their respective β-galactosidase fusion proteins. Lanes *S* have been probed with a 1:5,000 dilution of prototype serum SD7 and shows multiple banding. Lanes *1–8* have been probed with anti-p542-B (*left*) or anti-p554-B (*right*), either unabsorbed (lanes *1*) or absorbed with solid phase (lanes *2, 3, 5–8*), or soluble (lane 4) peptides. All peptides were at 100 μg/ml. Peptides used as absorbants: (*1*) None, (*2*) P62, (*3*) C2, (*4*) sC2, (*5*) P60, (*6*) E11, (*7*) F12, (*8*) P89 (see Table I). All lanes were developed for IgM antibodies.

To distinguish which bands were encoded by B cell DNA rather than by EBV DNA, the anti-p542-B and anti-p554-B were evaluated with the EBV-negative BJAB B lymphoblastoid cell line (Fig. 4). The major difference was that the 77-kD band (EBNA-1) seen in WiL2 cells by anti-p542-B was not seen in the EBV-negative BJAB line. Anti-p554-B showed both 72 kD and dye front proteins in both WiL2 and BJAB cells. (The two diffuse bands seen with the BJAB cells at 75–77 kD, but not with the WiL2 cells, are due to secretory and transmembrane mu-chains. These were seen directly by the developing reagent. They are not present in the WiL2 line we used, which is an Ig-deficient variant).

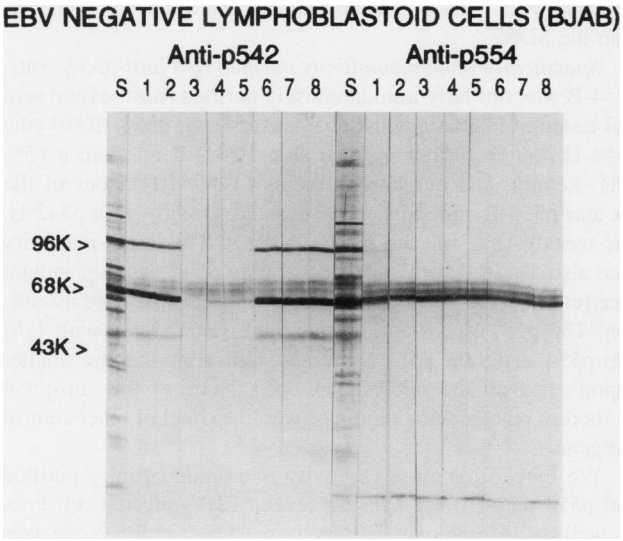

Figure 4. Reactivities and specificities of immunoaffinity-purified anti-p542-B and anti-p554-B autoantibodies in Western blots with whole cell extracts of the EBV-negative BJAB Burkitt cell line. See Fig. 3 for explanations.

Table I. Peptides of Interest

	Peptide	Source
p60	GGGAGAGGAGAGGGGRC	EBV EBNA-1
p62	AGAGGGAGGAGAGGGAGGAGC	EBV EBNA-1
p89	CRARGRGRGRGEKRPM	EBV EBNA-1
E11	KGGWFGKHRGQGGS	EBV EBNA-1
F12	IMSDEGPGTGNGLGEC	EBV EBNA-1
C2	SSSSAGGGGGGAGGGGGGGGSGG	CMV Early antigen
p542	GGGASGGGGGGGGSGGGGSGGG GGGGSS	B cell
Ker II	SSGGYGGGSSGGGGGGYGGG	Epidermis
Cytoker 1	GGFGGAGGFGGAGGFGGAGGF	Epidermis
Cytoker 10	GGGGFGGGGFGGGGIGGGGF	Epidermis

To determine whether any of the bands were related to the gly/ala autospecificity that we had previously observed in many infectious mononucleosis sera (11), we absorbed the immunoaffinity-purified antibodies with solid phase peptides. The peptides P60 and P62 (Table I) represent sequences in the gly/ala region of EBNA-1, P60 being at its COOH-terminal end; F12 is outside the gly/ala region at the N-terminal end of EBNA-1; and E11 and P89 are also outside the gly/ala region, but in the COOH-terminal half of the molecule. Also available was the peptide (C2) of a cytomegalovirus (CMV) early antigen. This peptide is the presumed basis for an autoantibody production in CMV-induced infectious mononucleosis, which is very similar to that of EBV-induced infectious mononucleosis (14, 21).

As illustrated in Figs. 3 and 4 (*left*), 100 μg of solid phase C2 (lane *3*), or P60 (lane *5*), per ml of anti-p542 autoantibody eliminated the ability of anti-p542-B to react with the B lymphocyte antigens. Soluble C2 (lane *4*) was equally effective. The same quantities of P62, F12, P89, and E11 in solid phase were each ineffective. Both the C2 and P60 beads were reexamined at 25, 50, and 100 μg of peptide/ml of anti-p542 eluate. There was no loss of inhibition at 50 μg, but the 77- and 71-kD bands were faintly visible at 25 μg (not shown). These results suggest that the reactivities of anti-p542 with each of the cellular proteins was related to its reactivity with the glycine rich 28-mer.

The anti-p554-B reactivity was distinctly different. None of the peptides in Table I inhibited the reactivity of the anti-p554-B with the B cell autoantigens, and the anti-p554-B did not distinguish between the EBV-positive and EBV-negative cells. Thus anti-p554-B sees different autoantigenic epitope(s) from that seen by the anti-p542-B, and it did not detect the EBV-encoded EBNA-1.

Anti-p542 and anti-p554 autoantibodies in IM and other sera. Patients with acute infectious mononucleosis showed significant elevations of both anti-p542 and anti-p554, as compared with sera from normal umbilical cord blood, from 16–17-yr-old normal students positive or negative for antibody to EBV

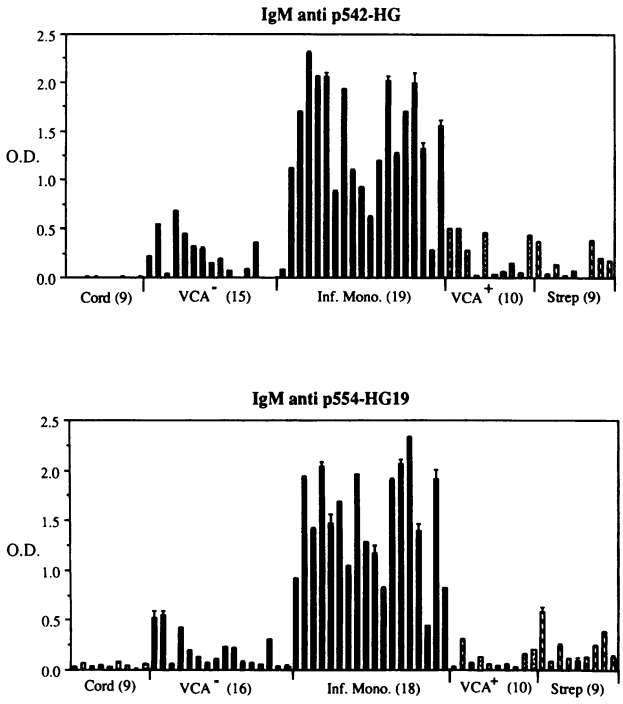

Figure 5. IgM anti-p542-HG and anti-p554-HG19 in sera from IM patients and controls by ELISA. All IM patients were in their acute illnesses, their sera having been taken at their first visit to the student health clinic. The strep group was a series of students similarly giving blood at their initial visit, but for acute strep throat, culture positive. The VCA+ and VCA− sera were obtained from local high school seniors, ages 16–17. Cord, cord blood sera.

viral capsid antigen, and from a series of students diagnosed to have acute streptococcal sore throats ($P < 0.0001$ for each) (Fig. 5). The sera were examined at 1/100, which is a dilution that is regularly on the downslope of their dilution curves. Among the controls, both autoantibodies were significantly lower in the cord blood sera than in the other control groups pooled ($P < 0.001$).

10 sera from infectious mononucleosis, 9 from patients with acute streptococcal sore throat, and 24 from normal controls were also studied with the p542-B and p554-B recombinants in Western blots. Bands were seen only with the sera of mononucleosis patients with high titers of anti-p542 or anti-p554, and these were of the expected mobilities for the p542-B and p554-B preparations used.

Immunoaffinity purified anti-p542 is not polyreactive, but binds diverse configurations in the gly rich epitope, including EBNA-1. We isolated the autoantibodies from multiple IM sera by solid phase absorption from p542-B protein on nitrocellulose strips, exactly as had been done (see above) with the SD7 serum. The eluates were then assessed in ELISA against the recombinant proteins p542-H and p554-H, the synthetic gly/ala peptides P60 and P62, peptide P89 (a negative control), dermal keratin, and an additional set of unrelated common antigens (Table II).

None of the immunoaffinity-purified anti-p542-B autoantibodies were reactive against the 8 unrelated antigens. However, 8 of the 10 reacted as strongly with keratin as they did with p542, a cross-reaction which we have previously recognized for IM autoantibodies (11) and which can be understood in terms of the specific sequences probably involved (Table I). Reactivity was also seen with synthetic gly/ala peptides in three patterns: for three eluates the reaction was better with P60 than with P62, for another four the reverse was true, and in one case there was no reactivity with either peptide, although this antibody still reacted with keratin. The signals given by the keratin were significantly greater than those given by the peptides in six of the eight. These relative differences in reactivity with keratin and the peptides held true in repeated analyses.

4 of the 10 anti-p542 eluates cross reacted with p554-HG19. This cross-reaction could not be attributed to the hexahistidine tail, since the eluates failed to bind HuHsp60-H which also possessed this tail. Nor could it be ascribed to common contaminants from *E. coli,* since the autoantibody activities were not reduced by preabsorption with an *E. coli* lysate, and since the nonreacting HuHsp60-H had been prepared from a nickel column, similarly, and would be expected to show similar contaminants, if any.

These results are taken to imply a minimum of four autoantigenic epitopes on p542. One is a non–glycine-rich epitope on p542 which is cross-reactive with p554, e.g., SD30, the presence of which is confirmed in the following paper (10). The other three are inferred from the three patterns of reactivity with P60, P62, and keratin (Table II) and must be related to its 28-mer glycine rich sequence. Considering the limited size of the 28-mer these are probably overlapping.

This evidence for multiple epitopes on p542 attests to precise and differing specificities for anti-p542 from serum to serum. A further manifestation of this can be seen in a variation in the reactivities of SD7 anti-p542-B with EBNA-1 from different cell lines (Fig. 6). It reacted with EBNA-1 in P3HR1 cells, but not in B95-8 cells. Additionally, although SD7 anti-p542-B reacted with EBNA-1 in WiL2 (Fig. 3), it did not react with the EBNA-1 in Raji cells (not shown).

An alternative conclusion, however, could be that anti-p542 does not see EBNA-1 at all, but only a protein that in some cells has the same behavior in Western blot analyses as EBNA-1. Therefore, we examined immunoaffinity-purified anti-p542-B with lysates of COS-7 cells taken before and after transfection with the EBNA-1 gene (Fig. 7). The autoantibodies were prepared from the sera SD 30, 41, and 55. All three showed clear reactivity with EBNA-1, the former two less intensely, however, than the SD55.

Specificities of immunoaffinity purified IgM anti-p554. Anti-p554-B was similarly immunoaffinity purified from several sera and tested in ELISA against p554-H, p542-H, p554-HG19 (the p554-H protein further purified as a 19-kD band from a 15% gel), keratin, and unrelated proteins (Table III). Four of the six anti-p554-B antibodies cross-reacted strongly with p542-H, one weakly, and one did not cross-react. This cross-reactivity (see also Table II) was retained when a p542 deletion mutant (see reference 10) lacking the gly rich repeat was used as antigen. The p554-HG19 antigen gave the same signal with IgM anti-p554 as did the p554-H antigen, indicating that the smaller peptide had all the relevant epitopes. None of the anti-p554 antibodies reacted with keratin or with the panel of other control antigens.

We looked for cross reactivity of immunoaffinity purified anti-p554 with EBNA-1, using several EBV-infected cell lines as well as recombinant EBNA-1 in COS-7 cells. None was found. We then examined the reactivities of IgM anti-p554 immunoaffinity purified from SD7, 30, 33, and 54 against extracts of the virus-producing P3HR1 and B95-8 B cell lines, with and without a 72-h induction with PMA and sodium buty-

Table II. Reactivities of Immunoaffinity-Purified IgM Anti-p542-B from Various Sera With p542-H and Other Antigens

	Serum sources									
	Infectious mononucleosis									
Antigens	SD38	SD7	SD29	SD60	SD55	IM14	SD34	IM20	SD41	SD30
p542-11	1.129±.081	0.366±.034	1.107±.021	0.813±.038	0.979±.047	0.915±.025	0.546±.005	0.384±.029	1.402±.017	0.528±.018
p554-11	0.016±.012	0.021±.014	0.086±.002	0.010±.005	0.032±.007	0.007±.003	0.369±.009	0.459±.013	1.035±.027	0.317±.009
Keratin	1.229±.023	0.223±.014	1.008±.014	0.757±.017	0.998±.022	0.802±.059	0.808±.028	0.272±.008	0.184±.011	0.023±.013
p60	0.020±.006	0.387±.015	0.281±.004	0.433±.012	0.419±.026	0.461±.015	0.164±.028	0.085±.008	0.142±.015	0.011±.003
p62	0.085±.008	0.051±.010	0.008±.004	0.662±.012	0.614±.014	0.246±.002	0.458±.018	0.188±.023	0.000±.007	0.002±.004
p89	0.000±.005	0.018±.002	0.010±.003	0.017±.014	0.004±.009	0.010±.003	0.014±.010	0.018±.008	0.000±.003	0.015±.005
Human collagen II	0.000±.010	—	—	0.000±.005	0.057±.134	—	0.000±.010	—	0.000±.005	0.000±.004
Human IgG	0.000±.014	—	—	0.003±.008	0.014±.009	—	0.022±.007	—	0.000±.026	0.019±.003
Thyroglobulin	0.000±.009	—	—	0.000±.005	0.013±.004	—	0.000±.004	—	0.000±.027	0.000±.015
Hu.Hsp60-II	0.000±.003	—	—	0.005±.024	0.005±.002	—	0.016±.006	—	0.000±.010	0.013±.003
groEL	0.000±.010	—	—	0.000±.003	0.040±.003	—	0.064±.011	—	0.000±.001	0.000±.001
Pneumococcus	0.000±.001	—	—	0.000±.001	0.003±.005	—	0.000±.005	—	0.000±.001	0.000±.001
Tetanus toxin	0.000±.007	—	—	0.001±.006	0.014±.010	—	0.026±.014	—	0.000±.005	0.000±.002
Actin	—	—	—	—	0.000±.002	—	—	—	—	—
Insulin	—	—	—	—	0.013±.007	—	—	—	—	—

The values given are OD (mean±SEM of triplicate determinations). The underlined values are arbitrarily selected, representing those that are higher than the mean + 10 SEM of the highest nonunderlined value.

rate. No new bands ascribable to EBV-encoded proteins could be identified by these autoantibodies.

Affinities of binding of anti-p542 and anti-p554. To estimate affinities of binding of the autoantibodies, we carried out inhibition studies. For anti-p542, we used keratin as inhibitor, since the p542 recombinant protein is not sufficiently soluble for the purpose. 50–150 ng/ml of immunoaffinity-purified IgM anti-p542 from six sera were preincubated with increasing quantities of keratin for 1 h at 37°C, and the mixtures then added to ELISA plates precoated with p542-HG protein at 1 μg/ml. 50% inhibition of the reactivities of four of the anti-p542 that had had strong direct reactions in ELISA with keratin (see Table II) occurred at 0.3–0.5 μg/ml ($5–8 \times 10^{-9}$ M) of keratin. The calculated mole ratio of inhibiting keratin to available IgM binding sites was about 10:1 (Fig. 8). For the remaining two anti-p542 (SD30 and SD41), which had had little or no detectable reactivity with keratin in ELISA, 100-fold higher concentration of keratin was only partially inhibitory.

Inhibition of binding by anti-p554 was carried out using p554-HG19 both as antigen in solid phase and as soluble inhibitor (Fig. 9). The autoantibodies from sera 9.5 and SD54 were immunoaffinity purified and used at 83 and 431 ng/ml, respectively. 50% inhibition occurred for serum 9.5 at 1.5–2.0 μg/

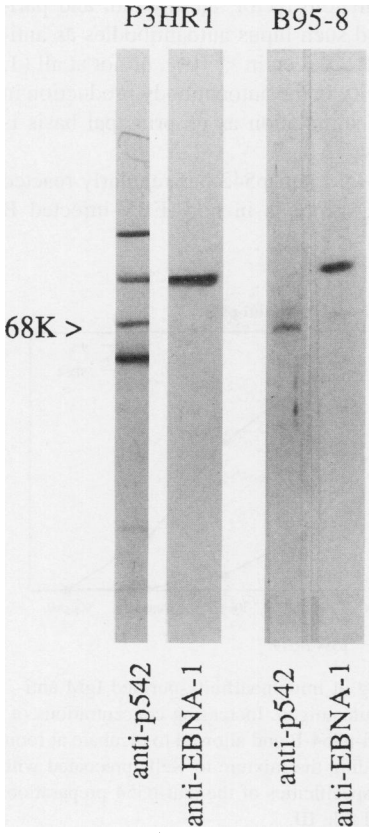

Figure 6. Differences in ability of anti-p542 to react with different EBNA-1 preparations. Anti-p542-B from SD7, or a standard anti-EBNA-1 antibody, was used to probe lysates of the P3HR1 Burkitt lymphoma cell or the B95-8 marmoset B lymphocyte in Western blots. The strips were developed with an anti-IgM reagent for the anti-p542, and an anti-IgG reagent for the anti-EBNA-1. The anti-p542 reacts with the Burkitt cell EBNA-1, but not with the marmoset cell EBNA-1.

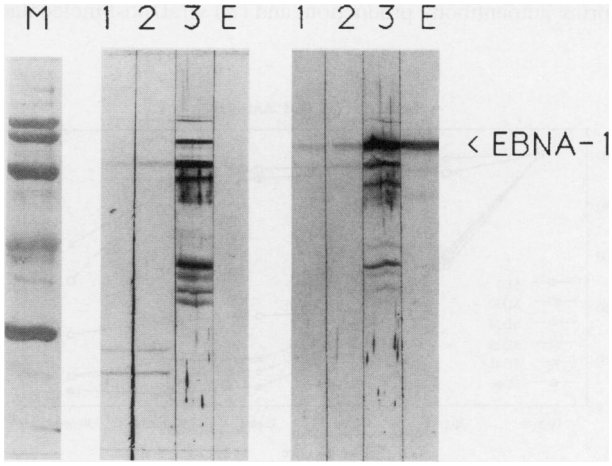

Figure 7. Reactivites of immunoaffinity-purified IgM anti-p542-B with nontransfected monkey kidney (COS-7) cells (*left*) and the same cells transfected with EBNA-1 (*right*). M, mol wts: 116, 97, 66, 45, and 29 kD. lanes *1, 2,* and *3* were probed with anti-p542 from sera SD30, SD41, and SD55. Lanes *E* were probed with a standard anti-EBNA-1 serum.

Table III. Reactivities of Immunoaffinity-Purified IgM Anti-p554-B from Various Sera with p554-H and Other Antigens

	Serum sources					
	Other	Infectious mononucleosis				
Antigens	9.5	SD54	SD7	SD29	SD30	IM3
p542-H	0.857±.050	1.086±.056	0.664±.021	0.051±.004	0.795±.130	0.243±.012
p554-HG19	1.149±.032	2.157±.034	2.321±.178	—	0.686±.056	1.386±.031
p554-H	1.196±.017	2.152±.032	2.238±.040	0.431±.036	0.558±.015	1.302±.032
Keratin	0.018±.011	0.021±.007	0.030±.008	0.030±.001	0.013±.008	0.007±.002
p60	0.030±.007	0.023±.007	—	—	—	—
p62	0.024±.013	0.020±.012	—	—	—	—
p89	0.060±.012	0.054±.015	—	—	—	—
Human collagen II	0.014±.004	0.005±.002	0.000±.002	0.006±.002	0.000±.004	0.004±.002
Human IgG	0.000±.015	0.000±.010	0.016±.007	0.021±.002	0.019±.003	0.017±.003
Thyroglobulin	0.006±.006	0.011±.010	0.000±.001	0.001±.003	0.000±.016	0.000±.001
Hu.Hsp60-H	0.000±.011	0.003±.015	0.000±.006	—	0.013±.003	0.000±.004
groEL	0.000±.008	0.007±.010	0.000±.005	—	0.000±.001	0.000±.003
Pneumococcus	0.013±.004	0.018±.004	0.000±.001	—	0.000±.001	0.000±.001
Tetanus toxin	0.024±.021	0.031±.031	0.006±.004	—	0.000±.002	0.000±.001
Actin	0.012±.017	0.019±.015	—	—	—	—
Insulin	0.029±.014	0.014±.033	—	—	—	—

The values given are OD (mean±SEM of triplicate determinations). The underlined values are arbitrarily selected, representing those that are higher than the mean + 10 SEM of the highest nonunderlined value.

ml of soluble inhibitor, or about 10^{-7} M, and at a 10-fold greater concentration of inhibitor for serum SD54.

Discussion

The basis for the prominent autoantibody production that characterizes infectious mononucleosis has been debated for years (1–9). Concepts considered have included: (*a*) polyclonal B cell stimulation by infecting EBV and consequent production of polyreactive autoantibodies from early B cells; (*b*) virus-specific host cell damage and resultant autoimmunization by released autoantigenic products; (*c*) the formation of specific viral/host protein complexes with a consequent adjuvant effect favoring autoantibody production; and (*d*) viral/host molecular homologies and consequent autoimmunization by molecular mimicry.

Prior studies have reported that autoantibodies are present to intermediate filaments in 70–97% of IM patients (2–7) and to lymphocyte outer cell membrane antigens in 37–46% (1, 8, 9). However, many other familiar autoantibodies such as antinuclear antibody, rheumatoid factor, antithyroid, and parietal cell autoantibodies, and such lupus autoantibodies as anti-Sm, anti-La, and anti-nRNP are seen in < 10%, or not at all (1, 2, 8). Thus there is selectivity in the autoantibody production in IM, and polyclonal B cell stimulation as its principal basis is no longer considered likely.

The p542 autoantigen. IgM anti-p542 has regularly reacted with 60-, 71-, and 104-kD proteins in non–EBV-infected B

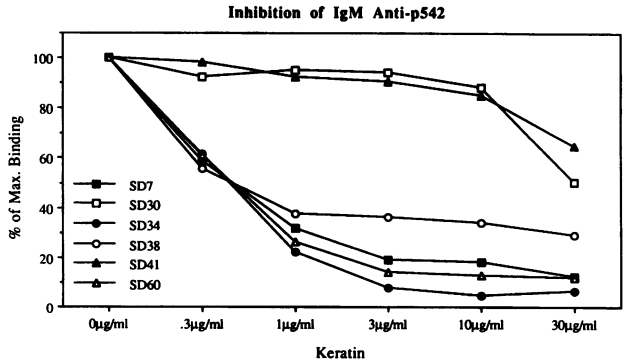

Figure 8. Inhibition of binding of immunoaffinity-purified IgM anti-p542 by preincubation with keratin. Increasing concentrations of keratin were added to anti-p542-B and allowed to incubate at room temperature for 1 h before adding the mixture to wells precoated with p542-HG at 1 µg/ml. The specificities of the anti-p542 preparations examined here are shown in Table II.

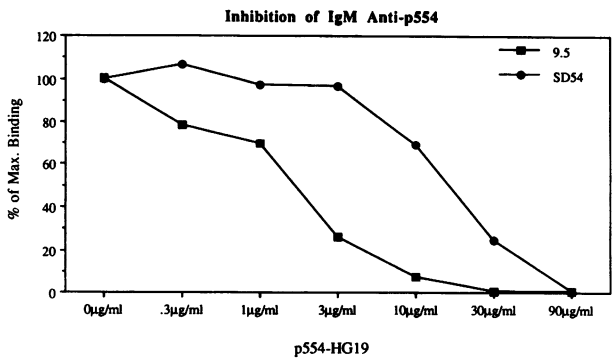

Figure 9. Inhibition of binding of immunoaffinity-purified IgM anti-p554 by preincubation with autoantigen. Increasing concentrations of p554-HG19 were added to anti-p554-B and allowed to incubate at room temperature for 1 h before adding the mixture to wells precoated with p554-HG19 at 1 µg/ml. The specificities of the anti-p554 preparations examined here are shown in Table III.

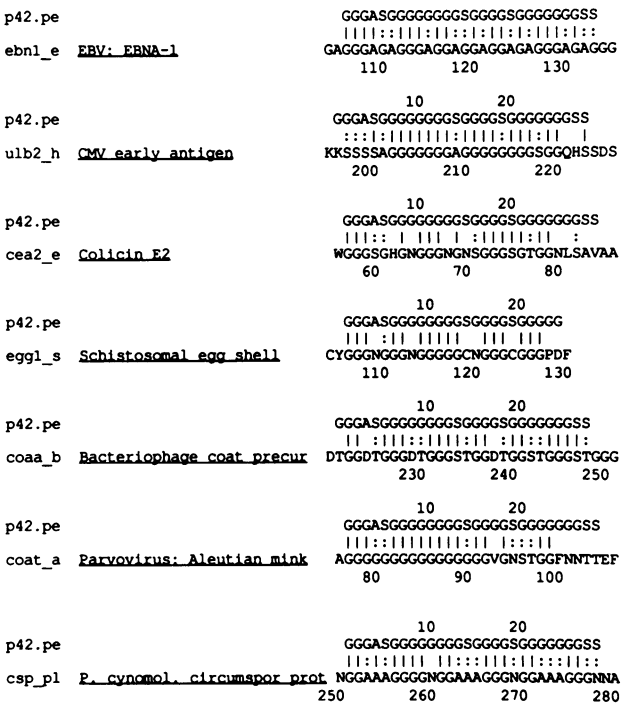

Figure 10. Glycine-rich microbial antigens with resemblance to p542. Selected from FastA analysis of SWISS-PROT database in the University of Wisconsin Genetics Computer Group program.

cells, one of which must be the p542 autoantigen itself. Since the 71-kD band was generally the strongest of the bands, developed earliest in Western blots, and was most uniformly present in cell lines other than B cells, it is likely that this is p542. The 60-kD band may be the same as a 62-kD protein reported by Luka et al. (22).

The glycine rich 28-mer, GGGASGGGGGGGGSGGGGSGGGGGGGSS, constitutes p542's major autoreactive site for IgM autoantibodies, as evidenced both from direct binding studies and from inhibition analyses. Immunoaffinity-purified anti-p542 reacted not only with keratin, which exhibits glycine rich sequences similar to the 28-mer in p542, but also with the EBNA-1 gly/ala peptide P60 or P62, or with both (Table II). It was inhibited by keratin (Fig. 8), and by P60 and a glycine rich peptide from a CMV antigen (Fig. 3 and 4). However, at least three different, but probably overlapping epitopes are implied for the 28-mer by the relative strengths of reaction that individual sera show for P60, P62, and keratin. Whether these epitopes are all linear, or include secondary or tertiary conformations, is not known.

Review of the GenBank and SWISS-PROT databases reveals that glycine rich sequences similar to p542's 28-mer are frequent among proteins of both mammalian and nonmammalian eukaryotics, and in several viral species (Fig. 10 and Table IV). These sequences have been thought to serve in some protein molecules as separators or spacers, or as hinges, between functionally different parts of the molecule (23, 24). In cytokeratins, in which they occur at the ends of rodlike molecules, they have been thought to be contact points for interaction with matrix protein (25); and in calcium-activated neutral protease a glycine rich sequence is associated with an interaction of the enzyme with cellular membranes (26). Thus there are multiple potential targets for an EBNA-1 induced autoimmunity, and it seems a certainty that other candidates will appear over time,

Table IV. Potential Autoantigens: Proteins Having p542-like Glycine Rich Segments

Human	Drosophila
Androgen receptor	Neurogenic protein mastermind
Keratin	Fork head protein
Homeobox 2.7	Double sex protein, male and female
Ca-activated neutral protease	Son of sevenless protein
Loricrin	Armadillo segment polarity protein
HLA Class II regular protein	Periodic clock protein
Atrial natriuretic peptide receptor	Protein tyrosine kinase SCR 28C
Heterogeneous RNP L	Regulatory protein ZESTE
RNA helicase	GABA receptor β precursor
Mouse	
Twist related protein	
Brain-2 POU-domain protein	

as more proteins with gly rich sequences are entered into the database.

The anti–p542 autoantibody response. The anti-p542 response would seem to derive, at least principally, from memory rather than naive or early B cells. Not only did the immunoaffinity-purified anti-p542 not display polyreactivity when tested against a variety of unrelated antigens, but it exhibited reasonably high affinity of binding with its autoantigen, as evidenced by inhibition by dermal keratin at $< 10^{-8}$ M. Additionally, there were significant, though modest, levels of anti-p542 in VCA negative adolescents, as compared to cord blood sera (Fig. 5). This difference is likely the result of preimmunization during antecedent infection by CMV, or by any of several other organisms known to produce proteins with gly rich sequences like p542's (see Fig. 10), and thus possibly capable of autoimmunizing in a manner similar to EBV.

The anti-p542 response in IM, however, can reasonably be attributed to EBV itself through molecular mimicry with EBNA-1. Anti-p542 reacts directly with EBNA-1, and this reactivity is inhibited by the gly/ala peptide P62. In similar findings, Garzelli et al. (27) isolated a monoclonal antibody from a patient with infectious mononucleosis that reacted both with EBNA-1 and in a cytoskeletal pattern in Hep2 cells; and Baboonian et al. (28) and Birkenfeld et al. (29), each showed cross reactivity of immunoaffinity purified anti-gly/ala antibodies with keratin. The mimicry between p542 and EBNA-1 is, however, complicated. EBNA-1 is recognized to have varying sizes in different cells, depending upon differences in the lengths of their gly/ala repeats, and we find preparations of anti-p542 that react with EBNA-1 from some cells but not others, e.g., Fig. 6. We presume that, among the variable EBNA-1's, configurations with which anti-p542 can react are also variably present.

Fine specificity differences are shown by anti-p542 from different individuals. This is most evident in the relative potencies of the autoantibodies in reacting with the P60 and P62 peptides, or with keratin. Whether these variations are based on differences in germ line V-region gene usage, differences in VDJ joining, accompanying T cell input and somatic mutation, or to some combination of these would be interesting to know. The first of these possibilities would assume that the fine speci-

ficity differences preexist in early B cells. However, the lack of polyreactivity (Table II) and the moderately high affinities we found for most of the anti-p542 preparations we studied (Fig. 8) argue for more mature antibody than this. Additionally, taking the IgM paraproteins in the sera of patients with Waldenstrom's macroglobulinemia to be neoplastic equivalents of early B cell antibodies, none of 50 sera had a paraprotein with anti-p542 specificity (data not shown). Also, cord blood serum specimens were negative in our assays. As to the other two possibilities for the fine specificity differences, variations in the third complementary determining region due to differences in VDJ joining as described by Martin, et al. (30) should be considered, but the lack of polyreactivity of the immunoaffinity-purified autoantibodies and their relatively high affinities again argue against this interpretation. It seems likely to us, therefore, that the fine specificity differences are based on somatic mutation from T cell input, perhaps nonspecifically derived (see Discussion, reference 10).

In addition to its glycine rich 28-mer, p542 has other autoantigenic epitope(s). This was first hinted at by the fact that two immunoaffinity-purified IgM anti-p542 preparations (SD41, SD30, Table II) showed strong autologous reactions with p542 but, in contrast to the anti-p542 from most of the other patients, little or none with keratin or the peptides. Keratin was not a good inhibitor of these anti-p542 preparations (Fig. 8). Additionally, SD41 and SD30, as well as two other anti-p542, cross reacted strongly with the otherwise unrelated p554. Confirmation of the existence of these additional epitope(s) has been achieved with deletion mutants that lack the glycine rich sequence (see reference 10). The existence of additional epitope(s) and the unlikelihood that all the epitopes would represent mimicry suggests to us that p542, which initially is probably an autoantigen solely by molecular mimicry, also may act as a true autoimmunogen in its own right, perhaps secondary to being complexed by antibody to the mimicking epitope.

Recognition that the principal specificity in anti-p542 is to a glycine rich sequence which cross reacts with keratin prompts a new consideration of the antikeratin autoantibody reactivity in IM sera. Antikeratin autoantibody has previously been interpreted (28) to indicate that EBV infection, which involves the epithelial cells of the oropharynx and probable epithelial cell damage, renders epithelial cytokeratin autoimmunogenic, either by extracellular spilling or by complexing with viral protein. This interpretation remains viable, but our findings present the alternative that antikeratin activity is, like the principal anti-p542 activity, based simply on cross reactivity with EBNA-1.

The p554 autoantigen. The most striking feature of the p554 recombinant protein was its propensity to bind multiple *E. coli* proteins during or after synthesis. This was repeatedly and prominently displayed, but only when the recombinant was expressed as the hexahistidine fusion protein (Fig. 2*B*). We did not see this when p554 was expressed as the larger β-galactosidase fusion protein, and we assume that the larger fusion partner occluded a binding site on the p554 molecule. We also did not see binding of bacterial proteins by two other hexahistidine fusion proteins we have prepared, p542-H and huHsp-H. These observations have led us to consider the possibility that p554 normally may have chaperonin-like activity, but this interpretation has not been formally examined.

Immunoaffinity-purified anti-p554 usually stains most intensely a singlet or doublet band at 26–28 kD in B lymphocytes. Additional bands with molecular weights of 58–60, 68, or 73 kD, and which are usually considerably less prominent than the 26–28 kD band, are variably detected with anti-p554 from individual sera. We have not ruled out the possibility that these lesser bands represent complexing of the 26–28-kD protein with other autologous proteins.

We know little about the autoantigenic epitopes on p554 other than that there must be more than one, as evidenced by the cross reactivity of anti-p554 with p542 in some sera but not others. Attempts to show cross-reactivity of immunoaffinity-purified anti-p554 with EBV-encoded antigens in PMA/butyrate-induced P3HR1 cells were unsuccessful, and thus an epitope with mimicry for an EBV protein is considered to be unlikely. Nevertheless, these in vitro studies may not adequately reflect the in vivo condition, and so this question can remain open.

The anti-p554 autoantibody response. Because of the propensity of p554 to complex with other proteins, we favor the possibility that p554 autoimmunizes through a schlepper effect (31). Like anti-p542, the anti-p554 response in IM is probably derived principally from memory rather than naive or early B cells, since there were significant, though low autoantibody titers in the VCA negative adolescent controls, the immunoaffinity-purified autoantibodies were not polyreactive, and the effective concentrations of inhibiting anti-p554 indicate binding affinities for anti-p554 greater than one would expect for autoantibodies from naive B cells.

Clinical considerations. To evaluate the possibility that IgM anti-p542 and anti-p554 autoantibodies are produced nonspecifically as a consequence of infection generally, rather than as a specific consequence of EBV infection, we tested sera taken from students during acute streptococcal sore throats and from 19 individuals who reported to the Student Health Clinic with symptoms suggestive of IM, but in whom the mono spot test was negative. Only three showed elevation of either of the two autoantibodies (data not shown).

The similarity between the glycine-rich sequence in p542 and the C2 sequence of a CMV early antigen asks whether anti-p542 could have been derived from prior CMV infection rather than from EBV infection. However, anti-CMV antibodies were found only in sera SD7, SD34, and SD61, and all were IgG rather than IgM. Thus the acutely elevated IgM anti-p542 autoantibodies in these EBV-induced IM sera were probably derived by immunization with EBNA-1 in most, if not all instances.

A further question is whether either of the two autoantibodies can be pathogenetically significant. Both detect intracellular antigens in T cells as well as in B cells, and variously also in nonlymphoid cells (data not shown), but we do not know their precise intracellular locations. Whether the autoantibodies can react with these autoantigens in vivo will depend upon whether, during the acute phase of the disease, they are exposed on the cell surface or spilled from damaged cells. Since there are apparently multiple autoantigenic epitopes on both p542 and p554 we believe it likely that they both can indeed be autoimmunogenic, and this suggests that spillage from cells during the acute illness does occur.

It is intriguing that the anti-p542 autoantibodies apparently derive from memory B cell pools in IM, but remain predominantly IgM in isotype in this illness. However, as noted in reference 10, IgG anti-p542 does occur in several autoimmune diseases, so in these circumstances the mechanisms that normally disallow switch of this autoantibody to IgG are often overcome.

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