Epstein-Barr Virus–induced Autoimmune Responses

II. Immunoglobulin G Autoantibodies to Mimicking and Nonmimicking Epitopes. Presence in Autoimmune Disease

John H. Vaughan,* Minh-Duc Nguyen,* Jean R. Valbracht,* Kevin Patrick,‡ and Gary H. Rhodes§

**Department of Medicine, Rheumatology Division, and the Sam and Rose Stein Institute for Research on Aging, University of California, San Diego, La Jolla, California 92093-0663; ‡Student Health Services, San Diego State University, San Diego, California 92182-0432; and §ViCal Corporation, San Diego, California 92121*

Abstract

During infectious mononucleosis, IgM autoantibodies are generated to a protein, p542, which contains a glycine-rich 28-mer epitope cross-reactive with the Epstein-Barr nuclear antigen-1 through Epstein-Barr nuclear antigen-1's glycine/alanine repeat. In normal individuals it is uncommon to find IgG anti-p542, but among patients with progressive systemic sclerosis, systemic lupus erythematosus, and ulcerative colitis high IgG anti-p542 (> 3 SD above the mean of normal 20–50 yr controls) occurred frequently. Lesser elevations occurred in Sjögren's syndrome, rheumatoid arthritis, ankylosing spondylitis, and Crohn's disease, but none with chronic hepatitis B infection. The reactive epitopes on p542 were mapped with deletion mutants, which indicated that the glycine-rich 28-mer was the major antigenic determinant, with lesser antibody responses to other epitopes. We conclude that normally there is an inability to generate IgG autoantibodies to the cross-reactive (mimicking) epitope of the p542 host protein, but that this inability is overcome in a proportion of patients with autoimmune disease. We conclude also that non–cross-reactive autoepitopes exist on p542 protein, to which IgG autoantibodies can commonly be formed in autoimmune disorders. The mechanisms responsible for the latter must involve different mechanisms than those responsible for autoantibodies to the mimicking epitope. (*J. Clin. Invest.* 1995. 95:1316–1327.)
Key words: autoimmunity • epitope • virus • scleroderma • lupus erythematosus

Introduction

Epstein-Barr nuclear antigen-1 (EBNA-1)[1] is a nuclear antigen encoded by EBV, and it is universally present in EBV-infected cells. It is critical to viral DNA replication and thus to viral persistence in host cells. It is also a potent antigen, its glycine/alanine (gly/ala) repeat constituting its major antigenic site, as well as a basis for autoantibody production by mimicry (1, 2). We have previously noted in infectious mononucleosis (IM) that IgM autoantibodies are produced to numerous B cell antigens, and that most of them are inhibited by gly/ala synthetic peptides representative of EBNA-1's gly/ala repeat (1). In the preceding paper (2), we reported that one of two recombinant B cell autoantigens recognized by IgM autoantibodies in IM, p542, contains a 28-mer sequence, GGGASGGGGGGGGSG-GGGSGGGGGGGSS, which is a mimicking epitope for the gly/ala repeat of EBNA-1. The 28-mer also is the major autoantigenic site in the p542 molecule. Similar Gly-rich epitopes exist in other mammalian and viral proteins, and these may constitute other targets or inducers of anti-p542-like autoimmunity.

Once infected by EBV, one carries the virus for the rest of one's lifetime (3). Since all cells infected by the virus produce the EBNA-1 antigen, EBNA-1 must pose a constant threat of autoimmunization. Here we follow the course of the antibody response to the gly/ala peptide of EBNA-1, together with the autoantibody levels to p542, in students during acute IM and through the following year. We also examine the frequency with which the autoantibody to p542 can be found in normal populations and in autoimmune diseases. IgM anti-p542 continues to be made long after acute IM. IgG anti-p542, however, develops infrequently during this time. IgG anti-p542 is also seen infrequently in normal controls, but it is found in high titer in subpopulations of several autoimmune diseases.

The appearance in multiple autoimmune diseases of IgG anti-p542, an autoantibody that can be attributed to virus infection and which is not explicable simply on the basis of polyclonal B cell stimulation, constitutes a novel finding.

In further studies, we have recognized two autoantigenic epitopes on p542 in addition to its mimicking 28-mer. Examination of one of these epitopes suggests that it has no mimicry with any EBV-encoded antigen, and thus that p542 can be an active autoimmunogen on its own. We suggest that the autoimmunogenicity of this second epitope depends upon preexisting autoantibody to the original mimicking epitope.

Methods

Sera. Students with infectious mononucleosis were volunteers at San Diego State University, San Diego, CA, who agreed to repeated bleedings during their acute illness and in a followup period. Diagnosis of IM was based upon the presence of typical clinical presentation, blood smears, and positive heterophil tests. Viral capsid antigen (VCA)-positive and VCA-negative controls were from 16–17-yr-old volunteer students in La Jolla High School, La Jolla, CA. The normal control popula-

Address correspondence to John H. Vaughan, Department of Medicine, University of California, San Diego, La Jolla, CA 92093-0663. Phone: 619-534-5442; FAX: 619-534-5399.

Received for publication 9 June 1994 and in revised form 5 October 1994.

1. *Abbreviations used in this paper:* CMV, cytomegalovirus; EBNA-1, Epstein-Barr nuclear antigen-1; gly/ala, glycine/alanine; IM, infectious mononucleosis; PSS, progressive systemic sclerosis; SjS, Sjögren's syndrome; UC, ulcerative colitis; VCA, viral capsid antigen.

J. Clin. Invest.

0021-9738/95/03/1316/12 $2.00
Volume 95, March 1995, 1316–1327

tions were composed of 29 healthy 20–50-yr-old volunteer hospital workers at Scripps Clinic and Research Foundation, La Jolla, CA, 6 similar volunteers from Cedars-Sinai Medical Center, Los Angeles, CA, an additional 37 from the UCSD Medical Center in San Diego, CA, and 30 healthy ≥ 70-yr-old volunteer Associates of the Sam and Rose Stein Institute for Research on Aging, UCSD. Sera of patients with SLE were provided by Drs. David Horwitz and Francisco Quismorio, University of Southern California Medical Center, Los Angeles, CA, Dr. John Harley, University of Oklahoma Medical School, Norman, OK, and Dr. Harry Bluestein, University of California, San Diego. Sera from patients with progressive systemic sclerosis were from Dr. Thomas Medsger, University of Pittsburgh Medical School, Pittsburgh, PA; Sjögren's syndrome (SjS) from Dr. Robert Fox, Scripps Clinic and Research Foundation; ulcerative colitis and Crohn's disease from Dr. Stephan Targan, Cedars-Sinai Medical Center; ankylosing spondylitis from Dr. David Yu, University of California, Los Angeles; Alzheimer's disease from Dr. Robert Katzsman, University of California, San Diego; and chronic hepatitis B infection from Dr. Frank Chisari, Scripps Clinic and Research Foundation. Sera from patients with rheumatoid arthritis were collected by ourselves from individuals with advanced, long-standing, active disease at Scripps Clinic and Research Foundation. Sera from patients with nondemyelinating neurological diseases were provided by Dr. Patrick Bray (4).

Enzyme linked immunoassays. ELISAs were carried out as previously described (2). In brief, 96-well microtiter trays (Costar Corp., Cambridge, MA) were coated with antigen or peptide at a previously determined optimal concentration (10 μg/ml), blocked with 1% bovine serum albumin, and probed with antibody applied in powdered milk for 1 h at room temperature. The secondary antibody was rabbit anti–human IgM or IgG conjugated with horseradish peroxidase. A 1:100 serum dilution was routinely used, having been shown to be regularly on the downslope of the titration curve and thus to provide a good measure of relative antibody concentration in various sera. For studies of antibodies to deletion mutants of p542, the wells were coated with mutant antigen at 5 μg/ml. Preliminary studies using the mutants at concentrations varying from 3 to 30 μg/ml revealed increasing OD values throughout this range, but only the antibody binding to wells coated at the lower antigen concentrations could be inhibited by soluble antigen at 30 μg/ml or less, and therefore 5 μg/ml was chosen for coating the wells to assay specifically for this inhibitable, higher affinity antibody population.

Western blots. Extracts of bacterial or mammalian cells in Laemmli buffer were prepared as previously described (2). They were electrophoresed in polyacrylamide gels, transferred to nitrocellulose, blocked with powdered milk in borate buffered saline, probed with purified antibody in powdered milk and 10% glycerol, and developed with enzyme-labeled goat anti–human IgG or IgM.

Recombinant autoantigens. The p542 and p554 autoantigens were prepared from the gene fragments, as described in a previous study (2). The β-galactosidase fusion protein, p542-B, was isolated as a 70-kD product after electrophoresis in large acrylamide gels of lysates of bacteria carrying the gene in a pSEM expression vector (2). The hexahistidine fusion proteins, p542-H and p554-H, were isolated on nickel columns from lysates of bacteria carrying the genes in pRSET expression vectors (Invitrogen, Inc., La Jolla, CA). The p542 gene fragment has been sequenced and is being reported separately (Rhodes, G. H., J. R. Valbracht, and J. H. Vaughan, manuscript in preparation).

Preparation of deletion mutants of p542. Deletion mutants of p542 were prepared using the p542 inserts in the pRSET expression vector, so that all mutants had NH_2-terminal hexahistidine fusion partners (Fig. 1). For the D1 mutant, the DNA was cut at an EcoNI restriction site in the insert and a HindIII site downstream in the plasmid, with subsequent blunt ending and religation. For the D2 mutant, the DNA was cut at the ClaI and EcoNI sites within the insert, with blunt ending and in-frame religation. For D3, the gene was cut at ClaI and the downstream HindIII site, with blunt ending and religation. JM109 bacteria were transformed with the respective mutant vectors and grown up with selection media. Expression of the mutant proteins was confirmed in polyacrylamide electrophoresis of the lysed bacteria. For initial evaluation of the reactivities of the mutant products with immunoaffinity-purified antibodies, crude lysates of the respective bacterial transformants were examined in Western blots. For later ELISA studies of serum antibodies, the mutant products were isolated from the lysates by two cycles of absorption and elution from nickel columns, the first elution being carried out and pH 4.0 and the second in 300 mM imidazole at pH 6.3, with final purification by FPLC.

p542 DELETION MUTANTS AND REACTIVITIES

	ClaI / EcoNI	IgM SD55	IgM SD41	IgG E90	IgG E77
p542	---------|---xxxxx---|--------	+++	+++	+++	++
D1	----------xxxxx---	+++	++	+++	+
D2	--------- --------	-	++	++	++
D3	---------	-	±	-	-

Figure 1. Diagrammatic display of the mutants prepared from the p542 gene, and the reactivities shown by four immunoaffinity-purified anti-p542 with recombinant products from the mutants. The mutants D1, D2, and D3 were prepared by ClaI/HindIII, ClaI/EcoNI, and EcoNI/HindIII cuts of p542, with blunt ending and religation (HindIII is downstream in the plasmid). xxxxx, Gly-rich sequence. The relative intensities of staining in Western blots shown at the right are also seen in Fig. 6.

Recombinant EBNA-1. Recombinant EBNA-1 was expressed by transfecting monkey kidney (Cos7) cells in 100-mm petri dishes, using an expression vector containing the SV40 early promoter and the EBNA-1 gene (2). The plasmid was kindly provided to us by Dr. Elliott Kieff (Harvard Medical School, Cambridge, MA).

Immunoaffinity purification of autoantibodies. Recombinant autoantigen was isolated on nitrocellulose strips after transfer from polyacrylamide gel electrophoresis. The excised strips were used for absorption of autoantibodies from serum, as previously described (2). The autoantibody was eluted at pH 11.5 and immediately neutralized in dihydrogen phosphate, 3% powdered milk, and 10% glycerol.

Antigens and synthetic peptides. The antigens were the same as those used in the previous paper (2). The synthetic peptides used here were EBNA-1 gly/ala (P62): AGAGGGAGGAGAGGGAGGAGC; EBNA-$1_{621-635}$: GDDGDDGDEGGDGDE; CMV EA (C2): SSSSAGGGGGAGGGGGGGSGG; and p542 21-mer: GGGGGGSGGGGSGGGGGGGSS.

Results

Lack of IgG anti-p542 during and after acute infectious mononucleosis. To evaluate the times of appearance of IgM and IgG anti-p542, and of antibody to a gly/ala peptide, P62, representing the mimicked region of EBNA-1, 14 volunteer college students with IM were bled during their acute illnesses and serially thereafter for up to 14 mo. All were heterophile antibody positive. The students remained ambulatory, returning at intervals to make blood donations during followup. Fig. 2, *top*, illustrates the levels of their IgM anti-p542 autoantibodies. These were high in the acute disease and fell somewhat by the third month, but they generally persisted elevated at about this level for the remaining period of observation. This persistence lasted well into the period when most patients were already showing a considerable rise in IgG antibody to the gly/ala (EBNA-1) peptide, P62 (Fig. 2, *bottom*).

Despite the rise in IgG anti-P62 peptide, IgG anti-p542 was infrequently seen (Fig. 2, *middle*). Two students had low levels of IgG anti-p542 during the acute episode, but both lost this by

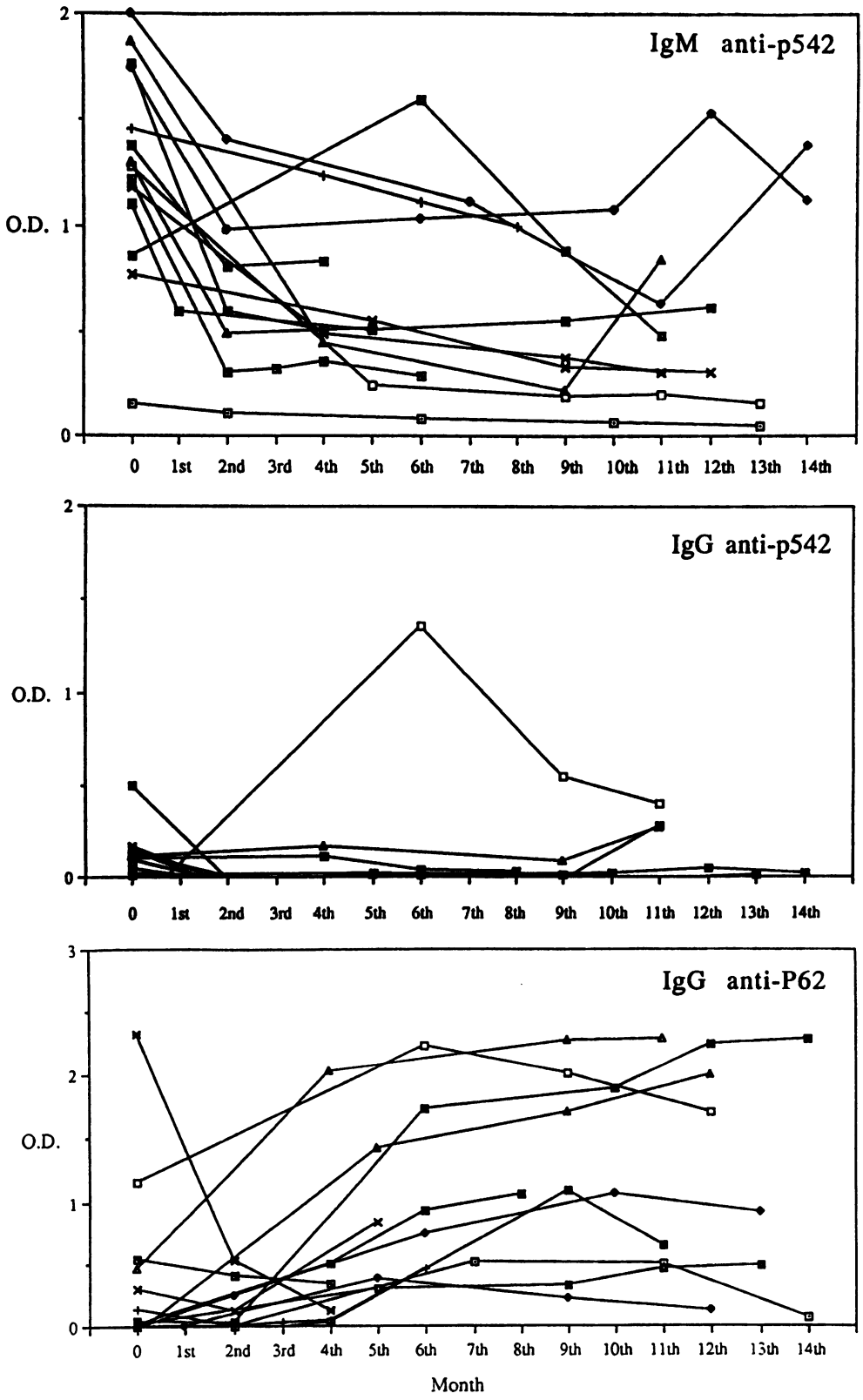

Figure 2. Serial observations of antibody levels by ELISA during and in convalescence from acute infectious mononucleosis. All patients were heterophile positive at initial diagnosis. The individual whose values peaked at 6 mo was the same in all three panels.

the first follow-up bleeding. Both of these students had very much higher IgM than IgG anti-p542 on these occasions. One student developed a striking elevation of IgG anti-p542 at the 6-mo follow-up, with a decline thereafter. This student also had a parallel rise and decline in his IgM anti-p542. Two other students showed small rises in IgG anti-p542 at about a year. On review of the student health clinic records, there were no remarkable symptomatic features, or routine clinical laboratory findings, to match these anti-p542 changes.

IgG autoantibody to a different and nonmimicking autoanti-

Table I. Autoantibody (Anti-p542) and Antibody (anti-p62 and Anti-C2) Titers in Autoimmune Diseases and Controls

		IgG Anti-p542H-G					IgG Anti-p62			IgG Anti-C2		
Serum sources	No.	Mean	SD	SEM	>2 SD	>3 SD	Mean	SD	SEM	Mean	SD	SEM
La Jolla control	29	0.036	0.069	0.013	1 (3.4%)	1 (3.4%)	1.143	0.552	0.102	0.087	0.127	0.023
San Diego control	37	0.014	0.044	0.007	1 (2.7%)	1 (2.7%)	1.264	0.640	0.105	0.021	0.023	0.004
L.A. control	6	0.055	0.126	0.051	1 (16.7%)	0 (0.0%)	1.509	0.250	0.102	0.028	0.061	0.025
≥70 yr control	32	0.049	0.170	0.030	2 (6.3%)	2 (6.3%)	1.498	0.332	0.059	0.051	0.162	0.029
Cord blood	9	0.110	0.253	0.084	1 (11.1%)	1 (11.1%)	0.741	0.573	0.191	0.000	0.000	0.000
VCA negative	15	0.000	0.000	0.000	0 (0.0%)	0 (0.0%)	0.050	0.112	0.029	0.000	0.000	0.000
VCA positive	24	0.068	0.070	0.014	2 (8.3%)	0 (0.0%)	1.274	0.846	0.173	0.050	0.155	0.032
Strept throat	9	0.011	0.020	0.007	0 (0.0%)	0 (0.0%)	1.148	0.586	0.195	0.000	0.000	0.000
IM	20	0.123	0.215	0.049	4 (21.1%)	4 (21.1%)	0.250	0.444	0.099	0.021	0.040	0.009
PSS (Pittsburgh)	40	0.440	0.721	0.114	15 (37.5%)	14 (35.5%)	1.784	0.379	0.060	0.533	0.818	0.129
Ulcerative colitis	12	0.419	0.691	0.199	4 (33.3%)	4 (33.3%)	1.010	0.717	0.207	0.204	0.585	0.169
SLE (Horwitz)	20	0.405	0.575	0.129	10 (50.0%)	9 (45.0%)	0.938	0.811	0.181	0.024	0.078	0.018
SLE (Harley)	86	0.214	0.438	0.047	24 (27.9%)	17 (19.8%)	1.015	0.627	0.068	0.078	0.287	0.031
SLE (Bluestein)	66	0.097	0.274	0.034	7 (10.6%)	6 (9.1%)	1.022	0.641	0.079	0.024	0.049	0.006
Sjögren's syndrome	49	0.256	0.528	0.075	13 (26.5%)	12 (24.5%)	1.094	0.686	0.098	0.098	0.251	0.036
Ankylosing spondylitis	12	0.227	0.260	0.075	4 (33.3%)	4 (33.3%)	1.474	0.216	0.062	0.021	0.032	0.009
Rheumatoid arthritis	37	0.225	0.456	0.075	9 (24.3%)	8 (21.6%)	1.271	0.558	0.092	0.039	0.111	0.018
Crohn's disease	12	0.190	0.378	0.109	3 (25.0%)	3 (25.0%)	1.243	0.633	0.183	0.112	0.320	0.092
Alzheimer	12	0.147	0.182	0.053	3 (25.0%)	1 (8.3%)	1.534	0.165	0.048	0.020	0.051	0.015
Hepatitis B	28	0.101	0.069	0.013	5 (17.9%)	1 (3.6%)	1.242	0.603	0.114	0.017	0.051	0.010
Non-MS neurological disease	15	0.085	0.169	0.044	1 (6.7%)	1 (6.7%)	0.841	0.497	0.128	0.005	0.009	0.002

Underlined means are significantly different ($p \leq 0.05$) from the La Jolla controls. The Alzheimer's disease means were not significantly different from the ≥70 yr controls.

gen, p554, (2) was elevated already at the first bleeding during acute IM in most patients (not shown), indicating that there was no general impairment in the ability to make IgG autoantibodies during the infection.

IgG anti-p542 in autoimmune diseases. The sera from VCA^+ and VCA^- 16–17-yr-old high school students, 20–50-yr-old healthy volunteer hospital workers, and 70–90-yr-old healthy volunteers had very little IgG anti-p542 reactivity (Table I and Fig. 3). By contrast, patients with progressive systemic sclerosis (PSS) and ulcerative colitis (UC), had strikingly elevated mean titers of IgG anti-p542; and patients with Crohn's disease, rheumatoid arthritis, Sjögren's syndrome, and ankylosing spondylitis had intermediate mean titers. Three separate series of SLE patients had high, intermediate, and low mean titers (discussed below). Patients with Alzheimer's disease, chronic hepatitis B infection, and nondemyelinating neurological diseases had the least antibody. The anti-p542 data are presented in Table I both as mean titers for each group, and as frequencies with which values ≥ 2 and ≥ 3 SD above the normal 20–50-yr-old controls were found.

In each of the autoimmune groups, many of the sera had IgG anti-p542 levels that actually were negative for the autoantibody, or within the normal range, but subgroups within each had very high levels. This is graphically demonstrated for PSS, SLE, and UC as compared with other groups in Fig. 3, and by the frequencies with which values ≥ 3 SD of the normal mean were seen in these and other autoimmune patients in Table I.

In previous studies (5), we noted that cytomegalovirus (CMV) infection can generate IgM autoantibodies with reactivities in Western blots resembling those of the IgM autoantibodies generated during EBV infection; and we have noted (Fig. 10 in reference 2) that CMV as well as other exogenous agents encode proteins which have glycine-rich sequences which are reasonable candidates for inducing anti-p542 autoantibodies. We wished, therefore, to get some estimate of whether either EBV or CMV might be associated with the IgG anti-p542. Therefore, we assayed by ELISA the titers of antibody to the EBNA-1 gly/ala peptide, P62, and the CMV early antigen peptide, C2 (Table I). Except for PSS, antibody to the CMV early antigen peptide was negative in almost all sera, there being no more than a single positive serum in each disease group. In PSS, however, significant anti-C2 titers occurred in 15/40 (38%) of the sera, the mean for the entire group being 0.533 OD. Within the PSS group there was no relation of the anti-C2 titer to the anti-p542 titer.

Anti-P62 was present in essentially all the patients' sera. Here too, there was no good correlation between the anti-P62 titer and the anti-p542 titer, except that for the vast majority of the sera the anti-p542 titer fell within the anti-P62 titer of the same serum (only 22 of 134 doubly positive sera had anti-p542 titer higher than anti-P62 titer, as opposed to a theoretical 50–50 distribution for unrelated antibodies, X^2 16.3, $P < .0001$, assuming equal sensitivities of the assays). This distribution is consistent with most anti-p542 autoantibodies being subcomponents of the anti-P62 antibodies. This possibility is more directly supported by data shown below in Figs. 4 and 7.

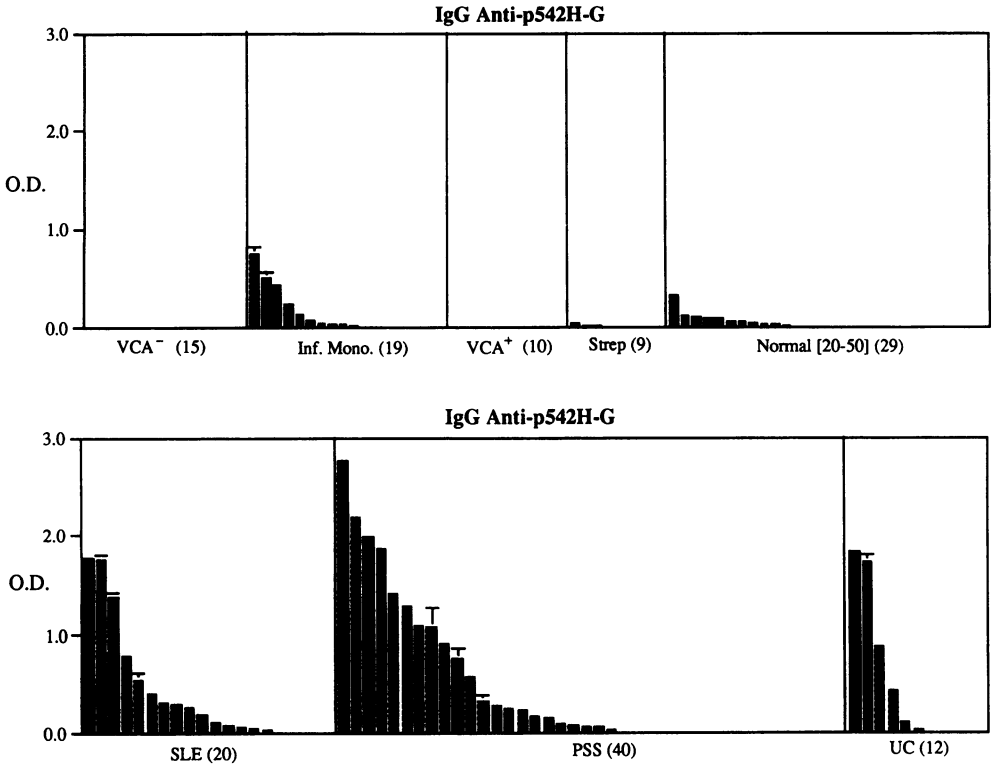

Figure 3. Titers of IgG anti-p542 by ELISA in normal VCA⁻ and VCA⁺ teenage controls, normal hospital employee volunteers 20–50 yr old, college students with infectious mononucleosis or strep throats, and patients with systemic lupus erythematosus, progressive systemic sclerosis, or ulcerative colitis. The SLE patients were from Dr. Horwitz (see Table I).

Specificity of IgG anti-p542. Seven SLE, one RA, two IM, and two normal sera were selected for immunoaffinity purification of their IgG anti-p542 autoantibodies. The autoantibodies were adsorbed onto p542-B, a 70-kD β-galactosidase fusion protein of p542 purified by gel separation and electrotransfer to nitrocellulose, as described in Methods. The autoantibodies eluted from this nitrocellulose-bound p542 were tested in ELISA against p542-H, a hexahistidine fusion protein of p542, which had been purified by adsorption and elution from a nickel column. All sera had been preabsorbed with extracts of *E. coli* carrying plasmids with antisense DNA inserts as a precaution against detecting IgG antibodies to *E. coli* products.

The immunoaffinity-purified IgG anti-p542 was highly specific (Table II). There was no cross-reactivity with keratin, which had been a major cross-reacting protein for IgM anti-p542 in our studies in the preceding paper (2), nor with any of the unrelated proteins and peptides tested, with the single exception of an anti-Hsp60 reaction in the normal serum AS47. Two of the seven SLE anti-p542 reacted with the P62 and P60 gly/ala peptides. Thus in ELISA the IgG autoantibodies were highly specific for p542, but in only two cases could they be clearly associated by this method with a gly/ala reactivity.

Anti-EBNA-1 reactivity of anti-p542. The weakness of the reactivity of anti-p542 with the gly/ala peptides in ELISA put in question whether anti-p542 does in fact cross-react with EBNA-1. We, therefore, examined more directly this presumed relationship by testing immunoaffinity-purified preparations of anti-p542 against recombinant EBNA-1. The EBNA-1 was pre-

Table II. Reactivities of Immunoaffinity-purified IgG Anti-p542-B from Various Sera with p542-H and Other Antigens

	Serum sources										
	Normal		R.A	IM		SLE					
Antigens	AS47	VCA + AG	IRA20	SD89	A1M1	E77	E90	E92	SLE13	SLE18	SLE19
p542-H	0.829±.009	0.621±.014	1.735±.078	1.133±.017	0.350±.028	2.024±.034	1.109±.020	0.880±.032	1.752±.053	0.637±.020	1.975±.023
p554-H	0.037±.002	0.075±.013	0.084±.007	0.058±.007	0.061±.009	0.009±.001	0.000±.008	0.000±.001	0.073±.010	0.062±.003	0.062±.003
Keratin	0.058±.011	0.036±.004	0.036±.007	0.055±.013	0.067±.009	0.000±.004	0.000±.014	0.000±.001	0.033±.004	0.055±.005	0.053±.017
p60	0.000±.008	0.060±.003	0.072±.009	0.077±.011	0.070±.013	0.006±.001	0.142±.019	0.012±.001	0.000±.011	0.027±.006	0.275±.005
p62	0.016±.011	0.051±.005	0.058±.008	0.048±.007	0.099±.010	0.016±.004	0.375±.018	0.003±.003	0.000±.007	0.041±.001	0.518±.007
p89	0.006±.040	0.137±0.24	0.130±.009	0.150±.022	0.161±.020	0.000±.001	0.000±.009	0.002±.006	0.024±.022	0.016±.006	0.007±.006
Human collagen II	0.000±.004	0.000±.004	0.000±.007	0.000±.002	0.000±.018	0.006±.018	0.021±.010	0.000±.018	0.000±.006	0.000±.005	0.000±.003
Thyroglobulin	0.139±.009	0.034±.008	0.000±.005	0.081±.006	0.021±.016	0.000±.008	0.000±.010	0.000±.005	0.015±.008	0.027±.007	0.044±.003
Human Hsp60-11	0.216±.028	0.163±.020	0.035±.005	0.150±.016	0.035±.011	0.000±.013	0.000±.010	0.000±.011	0.048±.011	0.074±.007	0.121±.020
groEl	0.011±.006	0.003±.003	0.138±.003	0.000±.004	0.199±.008	0.028±.011	0.000±.006	0.000±.011	0.023±.006	0.003±.002	0.005±.004
Pneumococcus	0.000±.003	0.000±.001	0.000±.001	0.000±.001	0.000±.005	0.000±.003	0.000±.011	0.000±.001	0.000±.008	0.000±.001	0.000±.001
Actin						0.000±.001	0.000±.008	0.000±.006			
Insulin						0.000±.002	0.000±.012	0.000±.004			

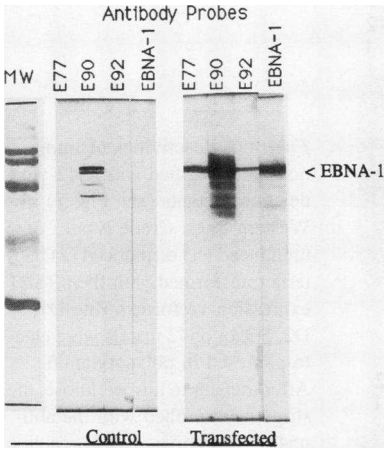

Figure 4. Western blot reactivities of immunoaffinity-purified IgG anti-p542 from SLE sera E77, E90, and E92 probed against an extract of COS-7 cells transfected with EBNA-1 DNA (*right*). Reactivities with an extract of nontransfected COS-7 cells is shown in the left panel. A standard anti-EBNA-1 antibody (G.R.) was used for positive identification in the lane marked EBNA-1. Mol wt (*MW*) markers are at 116, 97, 66, 45, and 29 kD.

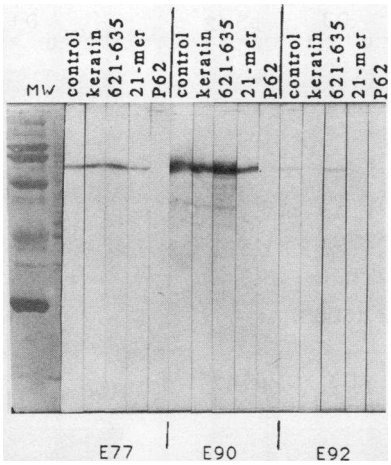

Figure 5. Inhibition of the reactivities of the IgG anti-p542 preparations illustrated in Fig. 4. Keratin, the EBNA-1 peptide 621–635 (see text), a peptide consisting of the C-terminal 21 residues of the p542 28-mer, and the Gly/Ala peptide P62 were preincubated at 10 μg/ml and 37°C for 1 h with the IgG anti-p542 preparations. These, with uninhibited controls, were then used to probe Western blots of the EBNA-1-transfected COS-7 cells. The first set of five lanes was probed with the inhibited and control anti-p542 preparations from E77, the second with similar preparations from E90, and the third from E92. Mol wt (*MW*) markers are the same as in Fig. 4.

pared from a lysate of monkey kidney cells (Cos7) transfected with the EBNA-1 gene (Methods). Extracts of the transfected cells were electrophoresed in polyacrylamide, transferred to nitrocellulose, and probed by the immunoaffinity-purified anti-p542. For controls, nontransfected monkey kidney cells were similarly grown, extracted, and submitted to Western blotting.

Presented in the left panel of Fig. 4 are Western blots with the control cells probed with the anti-p542; in the panel on the right are Western blots with the transfected cells. Lanes *EBNA-1* show the negative and positive reactions of the control and transfected cell preparations probed with a prototype IgG anti-EBNA-1 serum (G. Rhodes). Lanes *1–3* were probed with anti-p542 from three SLE sera and developed with an anti-IgG reagent. All three showed very strong reactivity with the recombinant EBNA-1. Additionally, the anti-p542 from E90 reacted with multiple bands in the nontransfected monkey kidney cells, indicating that autoantigenic configurations similar to ones in the human p542 are present also in this nonhuman primate.

Since anti-gly/ala reactivity had not been prominent in the ELISA data in Table I, the strengths of reactivities of the IgG anti-p542 with the recombinant EBNA-1 in Fig. 4 were at first unexpected. We, therefore, wondered whether these reactions might be based upon mimicking sequences between EBNA-1 and p542 that were additional to the gly/ala-28-mer mimic. We repeated the above Western blots using EBNA-1 as antigen, but preincubated the purified autoantibodies in (*a*) buffer control, (*b*) keratin, (*c*) a synthetic 15-mer peptide from EBNA-1 (residues 621–635) containing a pentamer DDGDE that is common to p542 and EBNA-1 and therefore potentially a second mimicking epitope, (*d*) a synthetic 21-mer peptide representing the downstream residues of the glycine-rich 28-mer in p542, or (*e*) the P62 gly/ala synthetic peptide of EBNA-1. The peptides were used in final concentrations of 10 μg/ml. The P62 peptide completely inhibited the reactions of all three of the autoantibodies with EBNA-1 (Fig. 5), and the 21-mer peptide of p542 was next most potent. Keratin was a weak inhibitor, and the DDGDE-containing peptide was completely inactive. Thus no additional epitopes could be demonstrated. IgG anti-p542 appears to react with EBNA-1 entirely through its gly/ala-28-mer cross-reacting specificity.

Multiple autoepitopes on p542. Although anti-p542 reacts with EBNA-1 purely on the basis of its anti-28-mer reactivity, additional autoantigenic epitopes were not ruled out by this. To look for other epitopes on p542, deletion mutants of the p542 gene were prepared. ClaI and EcoNI restriction sites are present on either side of the glycine-rich 21-mer in p542 (Fig. 1), and they are not present elsewhere in the gene or in the pRSET vector. These therefore were used for preparation of deletion mutants. D1 and D3 mutants were obtained by combining EcoNI and ClaI cuts, respectively, with a HindIII cut downstream in the multiple cloning site of the plasmid, then blunt ending and religation. D2 was a ClaI/EcoNI cut with an in-frame religation. All three mutants were very well expressed, giving excellent bands in Western blots when stained with amido black, and obtained in yields of 800, 255, and 690 μg from nickel columns with 50 ml starting vol of bacterial culture. At the right in the figure are depicted the three patterns of reactivity that we found in Western blots, using immunoaffinity-purified IgM or IgG anti-p542 to probe lysates of bacteria containing the deletion mutants.

Among nine IgG anti-p542 preparations from sera of patients with SLE, all showed reactivity with D1 and most with D2, as illustrated for E77 and E90 in Fig. 6, *bottom*. None showed reactivity with D3. All of four immunoaffinity-purified IgM anti-p542 preparations that had previously shown strong cross-reactivity with keratin (Table II in reference 2) reacted in Western blots exclusively with D1, as shown for SD55 in Fig. 6 *top* left. Both the SD30 and SD41 IgM anti-p542 that had reacted poorly or not at all with keratin (Table II in reference 2) reacted with both D1 and D2 (shown for SD41, *top right*). Both also reacted minimally with D3.

The deletion mutants were purified from the bacterial lysates by adsorption and elution from nickel columns and secondary FPLC to clear them of residual contaminating bacterial protein (as assessed with an anti-*E. coli* reagent, see Methods), and then used in ELISA to assay whole sera for autoantibody activity (Fig. 7). Most sera, whatever their source, that had previously exhibited IgG anti-p542 had IgG reactivity predominantly with the D1 mutant containing the 28-mer. IgG reactivity with the D2 mutant was seen in some sera, but almost always at a lesser titer than shown by the anti-D1 reactivity. IgG anti-D3 reactivity was least commonly seen.

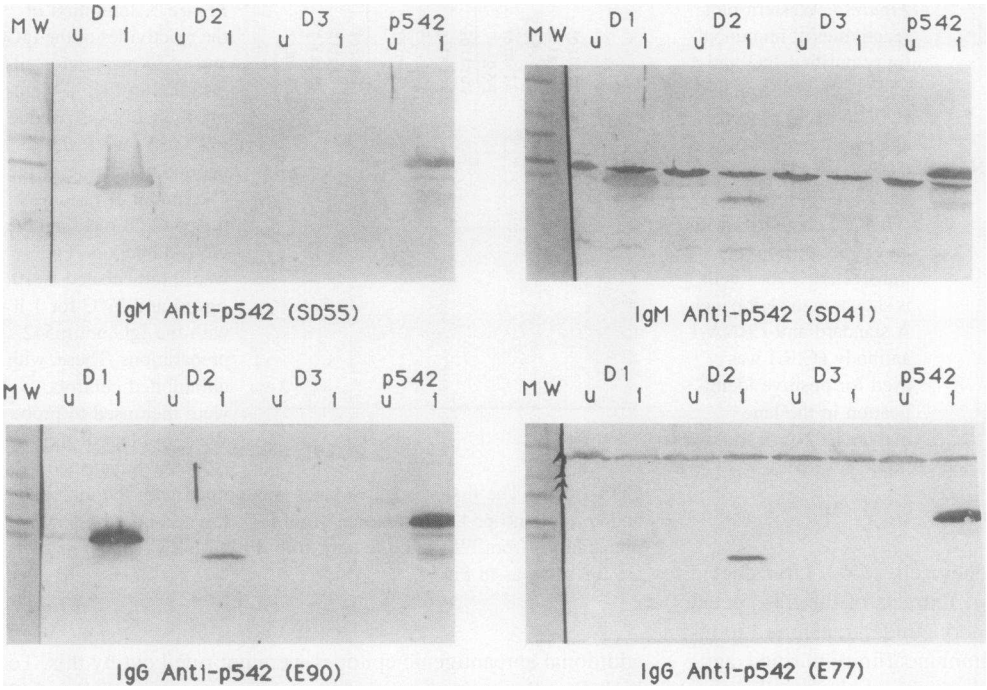

Figure 6. Reactivities of immunoaffinity-purified anti-p542 with deletion mutants (see Fig. 7) in Western blots. Crude lysates of uninduced (*u*) or induced (*i*) bacteria transformed with the pRSET expression vector carrying D1, D2, D3, or p542 inserts were electrophoresed in polyacrylamide. After transfer to nitrocellulose, the strips were probed with the anti-p542 preparations. Bands reactive across all lanes are due to anti-*E. coli* antibodies contaminating the anti-p542 preparations. SD55 reacted with D1 alone. E90 and E77 reacted with both D1 and D2. SD41 shows a very faint reaction with D3, as well as with D1 and D2. Mol wt (*MW*) markers were at 66, 45, 36, 29, and 24 kD.

Anti-D1, -D2, and -D3 during and after acute infectious mononucleosis. We reexamined the reactivities of the sera of the 14 patients shown in Fig. 1 during and after acute infectious mononucleosis for their reactivities with the deletion mutants. High levels of IgM autoantibody reactivity were seen to all three mutants (Fig. 8). The values were high initially and continued to be elevated, but at a somewhat lower level, throughout the follow-up. The strengths of the reactivities followed the general order D1 > D2 > D3. Except for SD30 and SD41, these IgM anti-D2 and anti-D3 reactivities had not been evident in Western blots (see Fig. 8). Since the ELISAs were carried out with whole sera and the Western blots with immunoaffinity-purified autoantibody, we interpret the difference to be due to the presence in the sera of low affinity as well as higher affinity autoantibodies, but with retention of only the higher affinity autoantibodies during the process of immunoaffinity purification.

In the teenage controls (see Table I and Fig. 2), there was also IgM reactivity for all three mutants, and this was true for some VCA^- as well as the VCA^+ sera (data not shown).

IgG autoantibody to the mutants was absent or in very low titer in the 14 IM patients. High titered anti-D1 occurred only in a single individual, and only transiently. This was the same individual who had shown the transiently elevated anti-p542 previously (Fig. 2). Lesser rises in IgG anti-D1 occurred at 12 mo in two other individuals, again the same two as for anti-p542 in Fig. 2. There was little or no IgG reactivity with D2 or D3.

Lack of mimicry of the D2 epitope with EBV antigens. IgG anti-D2, which was present in significant quantity in the SLE sera E77 and E90 (see Fig. 6), did not react with recombinant EBNA-1. This was clear from the inhibition studies in Fig. 4, in which the reactivities with EBNA-1 of the anti-p542 in both E77 and E90 were completely inhibited by P62. Thus anti-D2 does not react with EBNA-1.

To test whether anti-D2 cross-reacts with some other EBV-encoded antigen, we reacted immunoaffinity-purified anti-p542 from four sera known to have significant quantities of anti-D2 (one IgM and three IgG autoantibodies) in Western blots with lysates of P3HR1 and B95-8 B lymphocytes before and after their activation by PMA and butyrate to induce virus production. Lysates of the non–EBV-infected B lymphocyte BJAB, also before and after PMA and butyrate, were used as negative controls. The anti-p542 preparations were used as probes with and without inhibition by P62 or D2. In no case did we find the emergence of any new band reactive with anti-D2 in the activated lysates (data not shown).

Discussion

Consistent with our earlier experience in a general analysis of cross-reacting lymphocyte autoantigens by Western blots (1), IgG autoantibody to the recombinant protein p542 was usually very low or undetectable during convalescence from IM, even though IgG anti-gly/ala (anti-EBNA-1) antibodies non–cross-reactive with p542 developed abundantly. The systems that normally disallow switch to IgG of anti-gly/ala autoantibodies in convalescence from IM may include T cell suppressors, specific T cell cytotoxicity, downregulation of specific T cell receptor molecules, and suppression by antiidiotypic antibody or T cells (6–11), and also simple lack of development of anti-p542 specific T cells. Whatever the mechanism, B cells themselves must be central to the process, since the process involves recognition of very precise conformational specificities, i.e., those that differentiate Gly/Ala configurations that are and are not similar to ones present in the p542 28-mer. Antibody alone can do this.

As has been demonstrated for other autoantibodies (12–15), the anti-p542 response probably begins with polyreactive early B cells with low affinity sIgM. For anti-p542, the polyreactivity must include cross-reactivity between configurations in the gly/ala repeat of EBNA-1 and configurations in the Gly-rich 28-mer of p542. Expansion of B cells with this cross-

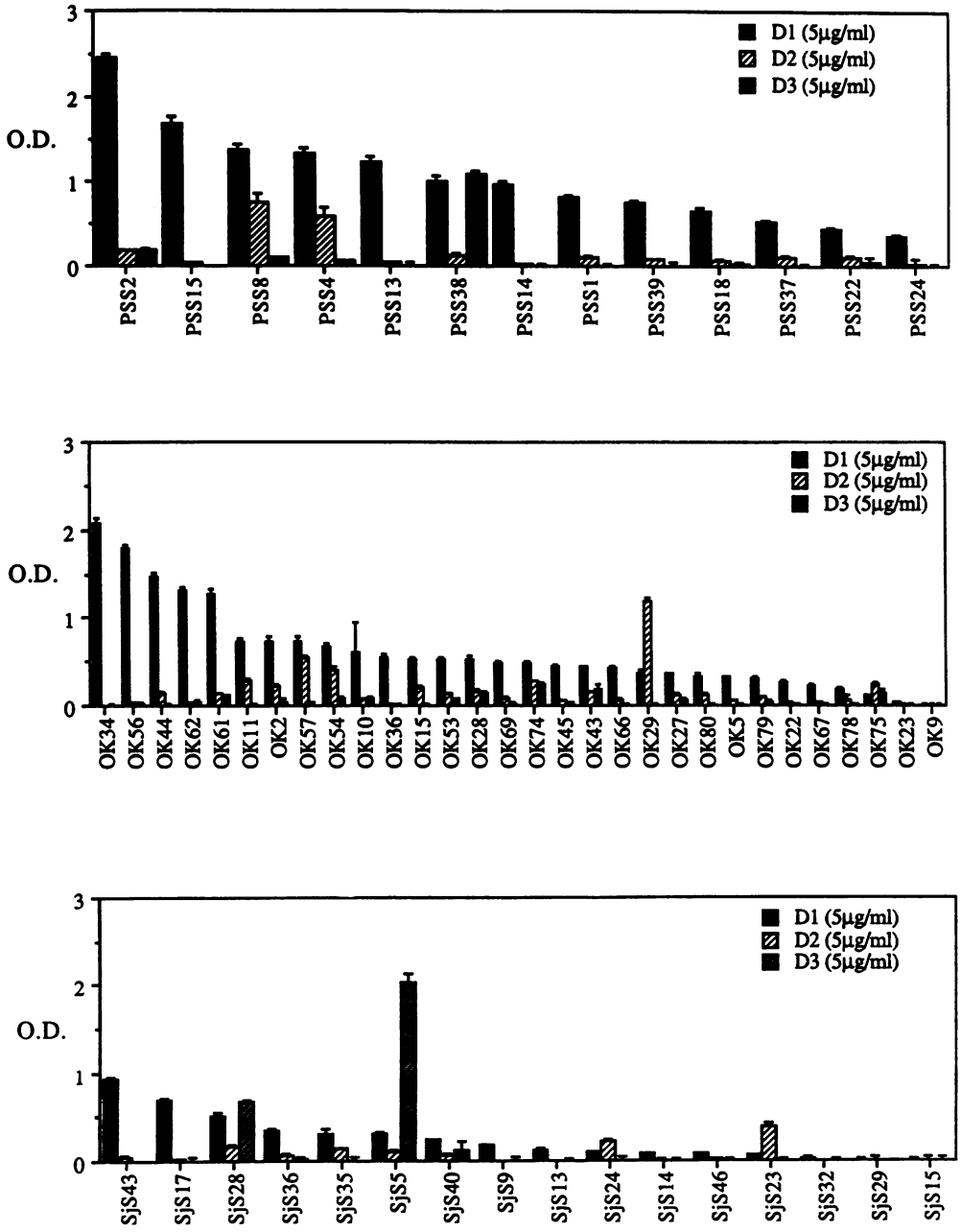

Figure 7. Reactivities in ELISA of anti-p542 positive sera from patients with *PSS*, systemic lupus erythematosus (*OK*), and Sjögren's syndrome (*SjS*) with the D1, D2, and D3 mutants of p542. To eliminate any possible anti-*E. coli* antibodies from the assay, all sera were preabsorbed with lysates of bacteria transformed with the pRSET vector carrying antisense inserts. The mutants were isolated by two cycles of elution from nickel columns (Methods), followed by FPLC, and used at 5 μg/ml to coat the ELISA plates.

reactivity—to give high IgM anti-p542 levels during acute IM—may occur initially by a T cell–independent, but EBNA-1–dependent immunization. (The mechanism for a T cell–independent B cell stimulation could be direct cross-linking of anti-gly/ala sIgM by the EBNA-1 gly/ala repeat. This repeat is poorly or not at all digested by tissue proteases, and it is long enough to display the same epitope several times. The entire P62 sequence, for instance, is present three times in the repeat, and shorter gly/ala sequences appear many more times than that.) The switch from IgM to IgG, however, would require T cell help, and this help must be given precisely to those B cells with p542/EBNA-1 cross-reactivity. Focus on this cell can be brought about only by the B cell's sIg binding the p542 autoantigen itself or, alternatively, EBNA-1 through those gly/ala epitopes of EBNA-1 that are cross-reactive with p542. Subsequent internalization of the p542 or EBNA-1, and presentation of their respective peptides, would allow for T cell response and stimulation of the B cells.

However, anti-EBNA-1–specific T cells have not been detectable in acute IM (16, 17); they emerge only later in convalescence. Anti-p542 specific T cells are probably also very infrequent, although this has not yet been directly investigated. So a strong system for generating an IgM→IgG shift for p542, either through EBNA-1 or through p542, probably does not exist during acute IM. Such a T cell deficiency may alone explain the infrequency of isotype shift, but the effect also could be to make the switch exquisitely sensitive to suppressor factors. Further studies will be needed to differentiate between these possibilities.

Multiple autoantigenic epitopes on p542. The p542 autoantigen has multiple autoepitopes, as is true also for other autoantigens (18–23). The principal epitope is the glycine-rich 28-mer,

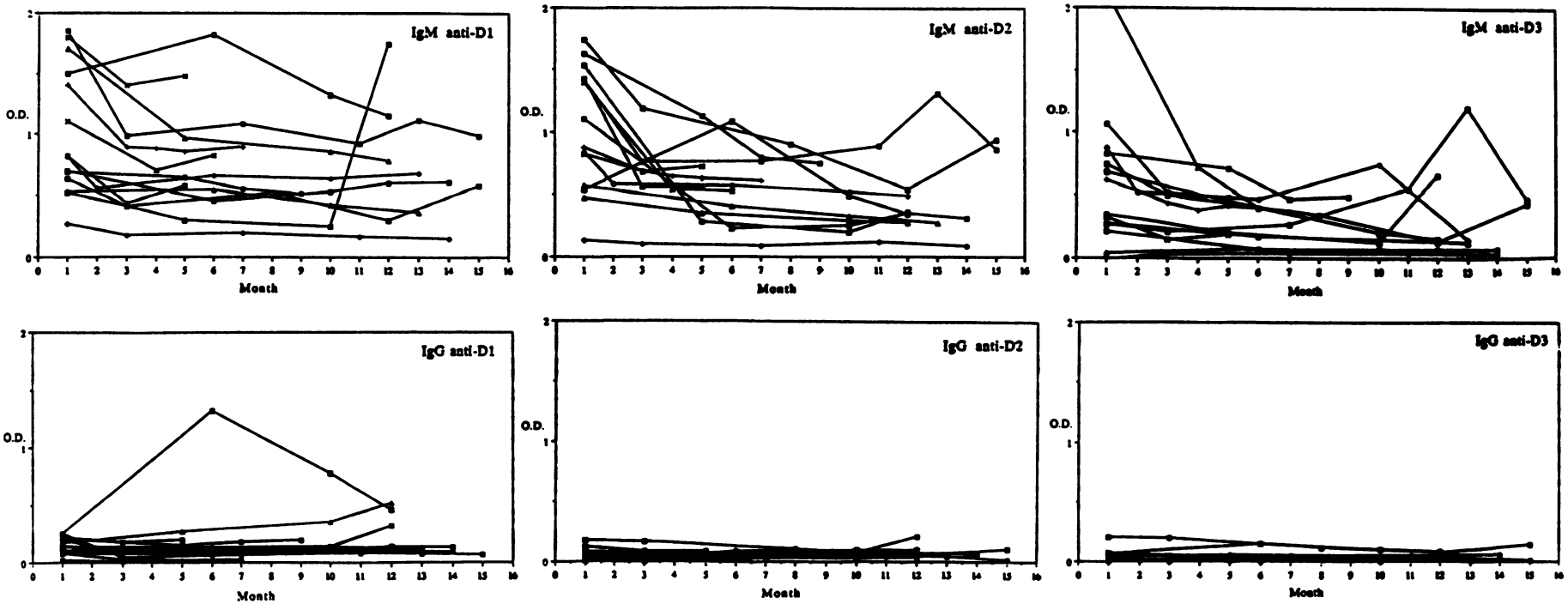

Figure 8. Serial observations on antibody titers to the D1, D2, and D3 mutants of p542 during and after acute infectious mononucleosis. The individuals are the same as in Fig. 2. The assays were by ELISA. The individual showing the high IgG anti-D1 at 6 mos is the same individual who showed a comparable peaking of IgG anti-p542 in Fig. 2.

which is in the D1 deletion mutant. When anti-p542 shifts to IgG, which generally occurs long after acute infection and often in conjunction with some autoimmune disease, the shift always includes autoantibody to the cross-reactive D1. It includes antibody to the non–cross-reactive epitope D2 in about half the sera and to D3 in about 10% of sera. This order in frequency follows the general order of the respective IgM titers of the autoantibodies in acute and convalescent IM sera (Fig. 8), and we interpret this to reflect the relative precursor frequencies for B cells carrying these reactivities.

The autoantigenicity of the D1 epitope is based on its mimicry with EBNA-1. The D2 epitope appears to be non–cross-reactive, neither with EBNA-1 nor with other EBV-generated proteins. Thus the mode of IgG anti-D2 induction must differ from that of anti-D1. The evidence we present here for its non–cross-reactivity is that anti-p542 sera containing IgG anti-D2 did not react detectably with recombinant EBNA-1 through the anti-D2 component (Figs. 4 and 5), nor with extracts of P3HR1 cells that had been induced to productive viral infection by PMA and sodium butyrate. Thus the development of IgG anti-D2 may be an example for autoantibodies of the epitope spreading described by Lehmann et al. (24) for autoreactive T cells in animals immunized with myelin basic protein.

We view that epitope spreading to D2 may operate in the following manner. IgG anti-D2 induction must depend initially upon precursor B cells with low affinity anti-D2 sIgM reactivity. Effective binding of p542 by these anti-D2–specific B cells will not likely be possible, however, except by complexing of the p542 molecule with antibody to the 28-mer, thus generating multivalency for D2. Consequent enhancement of binding, internalization, and presentation of p542 peptides to T cells, affinity maturation, and isotype switch would result. In this scheme, the development of non–cross-reactive anti-D2 is dependent on preexisting cross-reactive anti-D1. This scheme of anti-p542 production is susceptible to testing in an animal model, and it may prove generally descriptive of other autoantibody production.

The scheme assumes that p542 gets into the extracellular space, where p542 could bind anti-D2 reactive B cells. We have so far not detected p542 by flow cytometry as a surface antigen (not shown), and we have not tested for it in extracellular body fluids. There is precedent, however, among other intracellular autoantigens for their sometimes occurring extracellularly, i.e., both myelin basic protein and thyroglobulin have been directly demonstrated in the circulations of normal persons (25, 26). Other observations also are pertinent: (*a*) autoantibodies to intracellular antigens in autoimmune diseases are usually IgG in isotype, which most likely means that autoreactive B cells have picked up the autoantigen extracellularly and then themselves acted as autoantigen-presenting cells to T cells; (*b*) autoantibodies to intracellular as well as extracellular autoantigens exhibit mutational changes characteristic of antigen-driven immune responses (13, 27, 28), which also probably means presentation of extracellular autoantigen; and (*c*) the multiplicity of autoepitopes on most autoantigens is difficult to explain simply by mimicry and probably means that there are potentially autoimmunogenic epitopes on most of them.

The studies of Lin et al. (29), and Mamula et al. (30), strikingly illustrate the importance of extracellular autoantigen in the development of T cell autoimmunity in the mouse. These workers showed that, while human cytochrome *c* injected into mice can induce antibody cross-reactive with mouse cytochrome *c*, there was no accompanying T cell autoimmune response unless mouse cytochrome *c* was also included in the immunizing injections.

IgG anti-p542 in autoimmune diseases. Although IgG anti-p542 was seen infrequently, and only in low titer, in normal control populations, high titers were seen in subsets of several autoimmune diseases. The highest mean titers were in PSS and in UC. Varying mean titers occurred among three SLE groups; and the lowest values were in chronic hepatitis B infection, Alzheimer's disease and nondemyelinating neurological disease controls. We do not yet know definitively why the elevated anti-p542 autoantibody responses occur the in autoimmune diseases, but the skewing of the distribution of titers of anti-p542 towards those of anti-P62 suggests that these two are related to each other.

A simple interpretation that the anti-p542 in these diseases is based on polyclonal B cell activation is not adequate, since all the patient groups had comparable mean titers of antibody to both GroEL and pneumovax antigens, except RA and SjS which were lower (Vaughan, J. H., M.-D. Nguyen, and G. H. Rhodes, manuscript in preparation). Furthermore, the mean titers of these two antibacterial antibodies were not significantly different between sera with abnormally elevated anti-p542 titers ($>$ 2 SD above the normal mean), as compared to those below this level, within each patient group.

One possibility for the elevated anti-p542 levels is that EBV induces the disease in a proportion of patients in each autoimmune syndrome, and that the IgG anti-p542 is a marker of EBV induction in those patients. Alternatively, the IgG anti-p542 is not a marker of a specific inducing agent, but of the fact that each of the diseases has an impaired ability to suppress the expansion of EBV-infected B cells, as has been shown for several of them (31–33), with consequent autoimmunization by resident EBNA-1. A third possibility is that other microbial antigens cross-reactive with p542 may play inductive roles independent of EBV. Finally, the appearance of IgG anti-p542 may be determined by whether the individual's HLA class II molecules are capable of presenting the appropriate p542 peptides for T cell help.

PSS, which had the highest anti-p542 titers, is a disease that is characterized by antitopoisomerase 1, antinucleolar, anticentromere, and antifibrillarin autoantibodies, among others (34). Of these, fibrillarin has in it a glycine-rich 22-mer (35) with 77% identity to p542. We have not yet determined whether this homology is the basis for the high anti-p542 or high anti-C2. Neither EBV nor CMV has generally been considered to be an inducing agent in PSS, nor has any other infectious agent been so considered. However, in view of the close relationship that anti-p542 has to antibody to the EBNA-1 gly/ala peptide P62, and the independently elevated antibody to the CMV peptide C2, this tradition perhaps should be reexamined.

The number of sera of patients with ulcerative colitis we have examined is too small for conclusions to be drawn about our findings. Nevertheless, it is of note that Farmer et al. have reported high anti-CMV titers in UC blood (36), and Wakefield et al. described the detection by nested polymerase chain reactions of EBV and CMV DNA in 16 and 17, respectively, of 21 biopsies of UC colonic tissue (37). Our additional finding in this small series of high anti-p542 titers encourages further investigation in the area.

The three SLE series brings up other important considerations. Anti-p542 was elevated ($>$ 3 SD above the normal mean)

in subsets of all three series of patients more frequently than in the controls, but the numbers of individuals in these subsets varied greatly within each series. The Los Angeles sera were obtained from patients who were hospitalized because of active disease and who had high antinuclear antibody titers. The Oklahoma and San Diego sera were obtained from both inpatient and outpatient sources, and without regard to the activity or inactivity of their disease. The San Diego patients were predominantly Black and Hispanic, the Oklahoma patients predominantly European in origin. At present, we do not know which, if any, of these differences were determinant of the frequencies with which the elevated anti-p542 levels were found.

Nor do we know whether differences in environmental exposure were determinant in the SLE groups, including not only exposure to CMV or other non-EBV viruses, but also exposure to various strains of EBV with differing gly/ala chain length differences (38–40), which we have associated with different cross-reactive potentials (see Fig. 7, reference 2). Antibody titers to EBV antigens and peptides are elevated in SLE (41–47), however, and the elevations are highly selective among EBV antigens (41, 42), as well as exclusive of some other viral species (47). So the elevations of anti-EBV antibodies are not explicable simply on polyclonal B cell stimulation. It remains possible that EBV is a significant, but not exclusive, factor in initiating or compounding this disease.

Finally, we have not followed any of these SLE patients longitudinally to determine the effects of treatment, or variation in disease activity, on their anti-p542 titers.

Among the remaining patient groups, the universally low values of IgG anti-p542 in the chronic hepatitis series document that elevations of this autoantibody are not simply the result of chronic inflammation. The intermediate levels in Sjögren's syndrome, ankylosing spondylitis, and rheumatoid arthritis were, as with the PSS, UC, SLE, and SjS patient groups, each due to subsets of patients with high anti-p542 titers. The mechanisms leading to this development of subgroups, and the implications for pathogenesis in the autoimmunities, will be important areas for future exploration.

Acknowledgments

We are indebted to Drs. Francisco Quismorio, David Horwitz, John Harley, Harry Bluestein, Thomas Medsger, Robert Fox, Stephan Targan, David Yu, Robert Katzman, Frank Chisari, and Patrick Bray for their donations of sera from the various patient groups studied here, and to Dr. Eng Tan for helpful suggestions. We also thank Drs. Dennis Carson and David Pisetsky for critically reading the manuscript.

This work was supported in part by a grant (AR21175) from the National Institutes of Health.

References

1. Rhodes, G., H. Rumpold, P. Kurki, K. M. Patrick, D. A. Carson, and J. H. Vaughan. 1987. Autoantibodies in infectious mononucleosis have specificity for the glycine-alanine repeating region of the Epstein-Barr virus nuclear antigen. *J. Exp. Med.* 165:1026–1040.

2. Vaughan, J. H., J. R. Valbracht, M.-D. Nguyen, H. H. Handley, R. S. Smith, K. Patrick, and G. H. Rhodes. 1995. Epstein Barr virus-induced autoimmune responses. I. IgM autoantibodies to proteins mimicking and not mimicking Epstein Barr nuclear antigen-1. *J. Clin. Invest.* 95:1306–1315.

3. Epstein, M. A., and B. G. Achong. 1979. The Epstein-Barr Virus. Springer-Verlag New York Inc., New York. 459 pp.

4. Bray, P. F., J. Luka, K. W. Culp, and B. S. Schlight. 1992. Antibodies against Epstein-Barr nuclear antigen (EBNA) in multiple sclerosis CSF, and two pentapeptide sequence identities between EBNA and myelin basic protein. *Neurology.* 42:1798–1804.

5. Rhodes, G., R. S. Smith, R. E. Rubin, J. H. Vaughan, and C. A. Horwitz. 1990. Identical IgM antibodies recognizing a glycine-alanine epitope are induced during acute infection with Epstein-Barr virus and cytomegalovirus. *J. Clin. Lab. Anal.* 4:456–464.

6. Adelstein, S., H. Pritchard-Briscoe, T. A. Anderson, J. Crosbie, G. Gammon, R. H. Loblay, A. Basten, and C. C. Goodnow. 1991. Induction of self-tolerance in T cells but not B cells of transgenic mice expressing little self antigen. *Science (Wash. DC).* 251:1223–1225.

7. Rocha, B., and H. von Boehmer. 1991. Peripheral selection of the T cell repertoire. *Science (Wash. DC).* 251:1225–1228.

8. Hannestad, K., K. Andreassen, and G. Kristoffersen. 1992. Anti-idiotypic immune responses against adjuvant-free isologous IgM monoclonal antibodies and their augmentation by complex formation between IgM and albumin in bovine serum. *Eur. J. Immunol.* 22:321–327.

9. Dang, H., M. Fischbach, and N. Talal. 1985. Anti-idiotypic antiserum to monoclonal anti-Sm inhibits the autoantigen-induced proliferative response. *J. Immunol.* 134:3825–3830.

10. Tilkin, A. -F., G. Vinci, J. Michon, and J. -P. Levy. 1990. Autoreactive HLA-DR-specific autoreactive T-cell clones: possible regulatory function for B lymphocytes and hematopoietic precursors. *Immunol. Rev.* 116:171–181.

11. Roitt, I. M., P. R. Hutchings, K. I. Dawe, N. Sumar, K. B. Bodman, and A. Cooke. 1992. The forces driving autoimmune disease. *J. Autoimmun.* 5:11–26.

12. Naparstek, Y., J. Andre-Schwartz, T. Manser, L. J. Wysocki, L. Breitman, B. D. Stolla, M. Gefter, and R. S. Schwartz. 1986. A single germline V_H gene segment of normal A/J mice encodes autoantibodies characteristic of systemic lupus erythematosus. *J. Exp. Med.* 164:614–626.

13. Olee, T., E. W. Lu, D. -F. Huang, R. W. Soto-Gil, M. Deftos, F. Kozin, D. A. Carson, and P. P. Chen. 1992. Genetic analysis of self-associating IgG rheumatoid factors from two rheumatoid synovia implicates an antigen driven response. *J. Exp. Med.* 175:831–842.

14. Martin, T., S. T. Duffy, D. A. Carson, and T. J. Kipps. 1992. Evidence for somatic selection of natural autoantibodies. *J. Exp. Med.* 175:983–991.

15. Steinberg, A. D., A. M. Krieg, M. F. Gourley, and D. M. Klinman. 1990. Theoretical and experimental approaches to generalized autoimmunity. *Immunol. Rev.* 118:129–163.

16. Szigeti, R., L. Timar, L. Weiland, W. Henle, G. Henle, K. Hennessy, E. Kieff, D. Sulitzeanu, J. Dillner, and G. Klein. 1986. Epstein-Barr virus (EBV) antigen-specific leukocyte migration inhibition (LMI) in infectious mononucleosis (IM). *Clin. Immunol. Immunopathol.* 41:342–350.

17. Petersen, J., G. Rhodes, K. Patrick, J. Roudier, and J. H. Vaughan. 1989. Human T cell responses to the Epstein-Barr nuclear antigen-1 (EBNA-1) as evaluated by synthetic peptide. *Cell. Immunol.* 123:325–333.

18. Gohill, J., P. D. Cary, M. Couppez, and M. J. Fritzler. 1985. Antibodies from patients with drug-induced and idiopathic lupus erythematosus react with epitopes restricted to the amino and carboxy termini of histone. *J. Immunol.* 135:3116–3121.

19. Francoeur, A. M., C. L. Peebles, P. T. Gomper, and E. M. Tan. 1986. Identification of Ki(Ku, p70/p80P) autoantigens and analysis of anti-Ki autoantibody reactivity. *J. Immunol.* 136:1648–1653.

20. Ogata, K., Y. Ogata, Y. Takasaki, and E. M. Tan. 1987. Epitopes on proliferating cell nuclear antigen recognized by human lupus autoantibody and murine monoclonal antibody. *J. Immunol.* 139:2942–2946.

21. Bini, P., J. -L. Chu, C. Okolo, and K. Elkon. 1990. Analysis of autoantibodies to recombinant La (SS-B) peptides in systemic lupus erythematosus and primary Sjögren's syndrome. *J. Clin. Invest.* 85:325–333.

22. Pisetsky, D. S. 1991. Systemic lupus erythematosus. *Curr. Opin. Immunol.* 3:917–923.

23. Chou, C. -H., J. Wang, M. W. Knuth, and W. H. Reeves. 1992. Role of a major autoepitope in forming the DNA binding site of the p70 (Ku) antigen. *J. Exp. Med.* 175:1677–1684.

24. Lehmann, P. V., T. Forsthuber, A. Miller, and E. E. Sercarz. 1992. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature (Lond.).* 358:155–158.

25. Roitt, I. M., and G. Torrigiani. 1967. Identification and estimation of undergraded thyroglobulin in human serum. *Endocrinology.* 81:421.

26. Paterson, P. Y., E. D. Day, C. C. Whitacre, R. A. Berenberg, and D. H. Harter. 1981. Endogenous myelin basic protein-serum factors (MBP-SFs) and anti-MBP antibodies in humans. Occurrence in sera of clinically well subjects and patients with multiple sclerosis. *J. Neurol. Sci.* 52:37–51.

27. Shlomchik, M., M. Mascelli, H. Shan, M. Z. Radic, D. Pisetsky, A. Marshak-Rothstein, and M. Weigert. 1990. Anti-DNA antibodies from autoimmune mice arise by clonal expansion and somatic mutation. *J. Exp. Med.* 171:265–297.

28. Marion, T. N., A. L. M. Bothwell, D. E. Briles, and C. A. Janeway, Jr. 1989. IgG anti-DNA autoantibodies within an individual autoimmune mouse are the products of clonal selection. *J. Immunol.* 142:4269*a*. (Abstr.)

29. Lin, R.-H., M. J. Mamula, J. A. Hardin, and C. A. Janeway, Jr. 1991.

Induction of autoreactive B cells allows priming of autoreactive T cells. *J. Exp. Med.* 173:1433–1439.

30. Mamula, M. J., R. -H. Lin, C. A. Janeway Jr., and J. A. Hardin. 1992. Breaking T cell tolerance with foreign and self-co-immunogens. A study of autoimmune B and T cell epitopes of cytochrome c. *J. Immunol.* 149:789–795.

31. Tosato, G., A. D. Steinberg, and R. M. Blaese. 1981. Defective EBV-specific suppressor T-cell function in rheumatoid arthritis. *N. Engl. J. Med.* 305:1238–1243.

32. Tsokos, G., I. T. Magrath, and J. E. Balow. 1983. Epstein-Barr virus induces normal B cell responses but defective supressor T cell responses in patient with systemic lupus erythematosus. *J. Immunol.* 131:1797.

33. Shore, A., R. Klock, P. Lee, K. M. Snow, and E. C. Keystone. 1989. Impaired late suppression of Epstein-Barr virus (EBV)-induced immunoglobulin synthesis: a common feature of autoimmune disease. *J. Clin. Immunol.* 9:103–110.

34. Fritzler, M. J. 1993. Autoantibodies in scleroderma. *J. Dermatol. (Tokyo).* 20:257–268.

35. Aris, J. P., and G. Blobel. 1991. cDNA cloning and sequencing of human fibrillarin, a conserved nucleolar protein recognized by autoimmune antisera. *Proc. Natl. Acad. Sci. USA.* 88:931–935.

36. Farmer, G. W., M. V. Monroe, D. A. Fuccillo, L. H. Barbosa, S. Ritman, J. L. Serer, and G. L. Gitnick. 1973. Viral investigations in ulcerative colitis and regional enteritis. *Gastroenterology.* 65:8–18.

37. Wakefield, A. J., J. D. Fox, A. M. Sawyerr, J. E. Taylor, C. H. Sweenie, M. Smith, V. C. Emery, M. Hudson, R. S. Tedder, and R. E. Pounder. 1992. Detection of herpesvirus DNA in the large intestine of patients with ulcerative colitis and Crohn's disease using the nested polymerase chain reaction. *J. Med. Virol.* 38:183–190.

38. Hennessy, K., M. Heller, V. van Santen, and E. Kieff. 1983. Simple repeat array in Epstein-Barr virus DNA encodes part of the Epstein-Barr nuclear antigen. *Science (Wash. DC).* 220:1396–1398.

39. Hennessy, K., and E. Kieff. 1983. One of two Epstein-Barr virus nuclear antigens contains a glycine-alanine copolymer domain. *Proc. Natl. Acad. Sci. USA.* 80:5665–5669.

40. Billings, P. B., S. O. Hoch, and J. H. Vaughan. 1984. Polymorphism of the EBNA/RANA antigen in Epstein-Barr virus-positive cell lines. *Arthritis Rheum.* 27:1423–1427.

41. Petersen, J., G. Rhodes, J. Roudier, and J. H. Vaughan. 1990. Altered immune response to glycine-rich sequences of Epstein-Barr nuclear antigen-1 in patients with rheumatoid arthritis and systemic lupus erythematosus. *Arthritis Rheum.* 33:993–1000.

42. Ngou, J., H. Graafland, and M. Segondy. 1992. Antibodies against polypeptides of purified Epstein-Barr virus in sera from patients with connective tissue diseases. *J. Autoimmun.* 5:243–249.

43. Origgi, L., R. Perego, C. Hu, E. Bertetti, P. D'Agostino, R. Asero, and P. Ribold. 1988. Anti Epstein-Barr virus antibodies in systemic lupus erythematosus. *Bolletino dell Istituto Sieroterapico Milanese.* 67:116–122.

44. Ngou, J., M. Segondy, J. M. Seigneurin, and H. Graafland. 1990. Antibody responses against polypeptide components of Epstein-Barr virus-induced early diffuse antigen in patients with connective tissue diseases. *J. Med. Virol.* 32:39–46.

45. Yokochi, T., A. Yanagawa, Y. Kimura, and Y. Mizushima. 1989. High titer of antibody to the Epstein-Barr virus membrane antigen in sera from patients with rheumatoid arthritis and systemic lupus erythematosus. *J. Rheumatol.* 8:1029–1032.

46. Kitagawa, H., S. Iho, and T. Hoshino. 1988. Detection of antibodies to the Epstein-Barr virus nuclear antigens in the sera from patients with systemic lupus erythematosus. *Immunol. Lett.* 17:249–252.

47. Phillips, P. E. 1975. The role of viruses in systemic lupus erythematosus. *Clin. Rheum. Dis.* 1:505–518.