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Immunoglobulin G subclass distribution of autoantibodies in systemic sclerosis, primary biliary cirrhosis, and overlap syndromes

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SUMMARY The IgG subclass reactivities of six anticellular antibodies were measured by indirect immunofluorescence on HEp_2 cells using murine monoclonal antibodies to the four human IgG subclasses. Patients with scleroderma, primary biliary cirrhosis (PBC), systemic lupus erythematosus, and mixed connective tissue disease were studied. Anticentromere antibody (ACA) was virtually all IgG1 and 3; antibody to multiple nuclear dots (NSpI) was IgG1, 2, and 3; antimitochondrial antibody was mainly IgG2 and 3; nucleolar staining was varied in subclass reactivity but most often IgG4; the diffusely grainy staining associated with Scl-70 antibody was chiefly IgG1; and the speckled pattern associated with anti-RNP antibody was always IgG1 and 4, with IgG2 and 3 in some cases. These data fail to support the hypothesis that the various patterns of autoimmune disease reflect differences in the biological properties of the associated antibodies. The prominence of IgG2 in antibodies associated with PBC suggests the possibility of an immune response independent of T cells in that condition. Differential subclass staining showed an unexpectedly high frequency of antibody to multiple nuclear dots in ACA positive sera, and such patients (all with CREST syndrome) could be at increased risk of developing PBC later.

Key words: antinuclear antibody, anticentromere antibody, antibody to multiple nuclear dots, Scl-70, (U1)RNP, scleroderma, CREST syndrome, systemic lupus erythematosus, mixed connective tissue disease.

Autoantibodies directed against certain intracellular antigens show remarkable, though not absolute, disease associations.[1] The IgG subclass distribution of some antinuclear antibodies (ANA) is of restricted heterogeneity.[2–10] It has been suggested that this might determine the biological activity of ANA, thereby influencing the pattern of antibody mediated tissue damage and the form of disease expressed. It has been argued, for instance, that anti-double-stranded deoxyribonucleic acid (anti-dsDNA) antibody is particularly associated with renal involvement in systemic lupus erythematosus (SLE) because there is a predominance of IgG1 and IgG3 antibodies[6 8 10 11] that fix complement.[6 11–13] The relative lack of renal and neurological disease in patients with anti-(U1)RNP antibody[14] has been attributed to a predominance of non-complement-fixing IgG2 antibodies,[8] though this is disputed.[10]

Serum ANA specificities in systemic sclerosis differ from those in SLE,[15 16] and some of these ANA are associated with patterns of clinical and serological involvement. For example, anticentromere antibody (ACA) is associated with the relatively benign CREST (calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly, telangiectasia) syndrome[15 17] and a lower prevalence of circulating immune complexes,[18 19] whereas antibodies to the Scl-70 antigen, recently shown to be topoisomerase I,[20] are associated with pulmonary involvement[21] and a higher prevalence of circulating immune complexes.[19] ANA are also a frequent finding in PBC, where ACA is again associated with features of CREST syndrome[22] and antibody to multiple nuclear dots (MND) is associated with sicca syndrome.[23] It is not known why particular ANA specificities tend to associate with different patterns of disease, but one possible explanation would be

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that there are differences in the IgG subclass distribution of the various ANA leading to differences of biological activity analogous to the situation proposed for SLE.

To investigate this hypothesis, indirect immunofluorescence was used to determine the IgG subclass distribution of six autoantibodies. The findings in systemic sclerosis were compared with those for primary biliary cirrhosis (PBC) and other connective tissue diseases. In the execution of these studies we also found that indirect immunofluorescence using anti-IgG subclass antibodies can sometimes be of value in clarifying complex autoantibody patterns.

Materials and methods

SERA

The sera studied were selected for the presence of particular antinuclear antibodies: anticentromere antibody in 50 cases, antibody to multiple nuclear dots in two cases, anti-Scl-70 antibody in eight cases, and anti-(U1)RNP antibody in 13 cases. The underlying diagnoses in these patients were scleroderma of the CREST variety in 40 cases (all ACA positive), diffuse systemic sclerosis in eight cases (selected for anti-Scl-70 antibody, all eight sera giving diffusely grainy ANA staining), primary biliary cirrhosis in 12 cases (with ACA in 10, multiple nuclear dots (MND, also termed NSpI) pattern in the remaining two, and antimitochondrial antibodies (AMA) in 11 of the 12). The sera containing anti-(U1)RNP antibody all gave typical speckled immunofluorescence and were derived from patients with diagnoses of systemic sclerosis (eight cases), systemic lupus erythematosus (two cases), and mixed connective tissue disease (three cases). These diagnoses were made by conventional criteria, including liver biopsy in the case of PBC.

IMMUNOFLUORESCENCE

Detection of autoantibodies by immunofluorescence was performed using HEp_2 cells (Ortho Diagnostic Systems, New Jersey) as substrate. Sera were diluted to 1/40 in phosphate buffered saline (PBS)

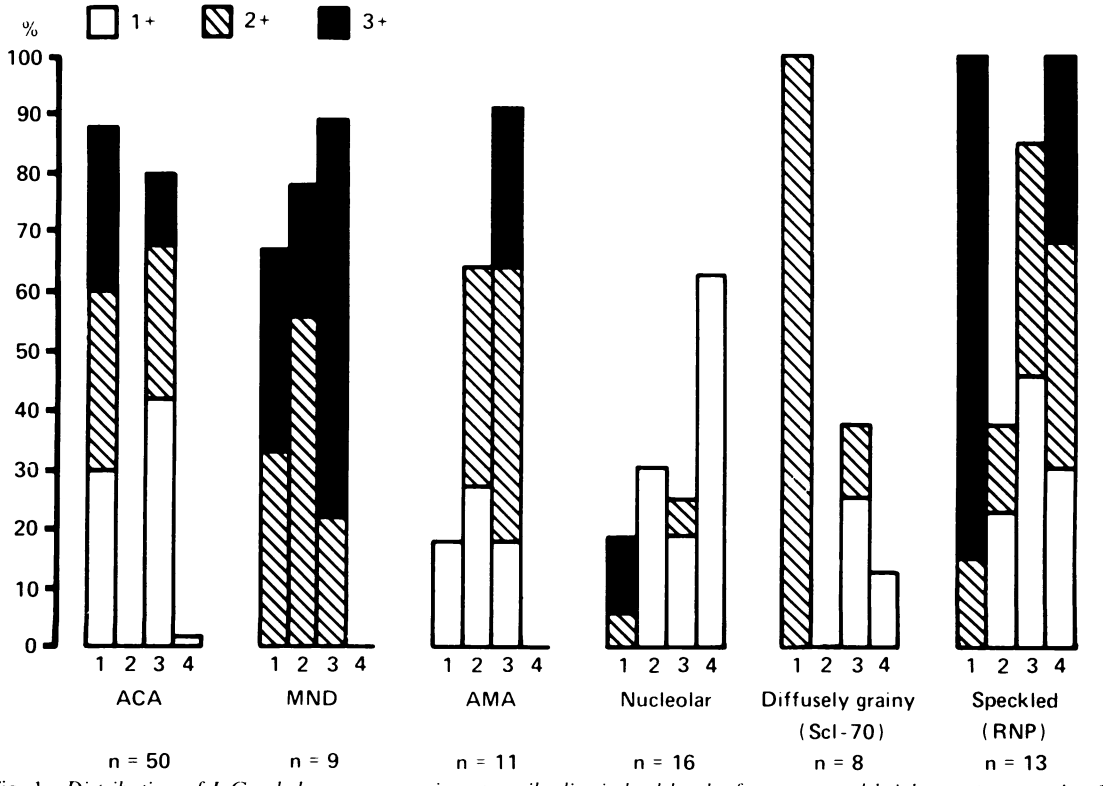

Fig. 1 *Distribution of IgG subclasses among six autoantibodies judged by the frequency and brightness (on a scale of 1+ to 3+) of the immunofluorescence on HEp_2 cells. (Reproduced with the permission of the University Department of Medical Illustration, Royal Infirmary, Manchester M13 9WL, England.)*

and incubated on cells for 30 minutes at room temperature. Slides were washed in PBS and overlayed with mouse monoclonal anti-IgG subclass antibodies (Seward, UK) for 30 minutes at room temperature. The optimal dilution of monoclonal antibodies in PBS had been determined to be 1/10 000 for anti-IgG1 (JL 512) and anti-IgG3 (ZG4), 1/5000 for anti-IgG4 (RJ4), and 1/500 for anti-IgG2 (GOM 2); there was no non-specific immunofluorescence with normal serum and saline controls. Slides were washed in PBS and overlayed with fluorescein conjugated sheep antimouse IgG (Serotec Ltd, UK) for 30 minutes at room temperature, and, after washing in PBS, coverslips were mounted in 50% glycerol in PBS. Immunofluorescence was read at a magnification of 1/500, defining patterns according to Bernstein *et al*[15, 23] and grading the intensity of fluorescence on a scale of 0–3.

IMMUNODIFFUSION

Anti-(U1)RNP and anti-Scl-70 antibodies were detected by double immunodiffusion using reference sera as described previously.[19] Only one precipitin line was detected in each serum selected for this study.

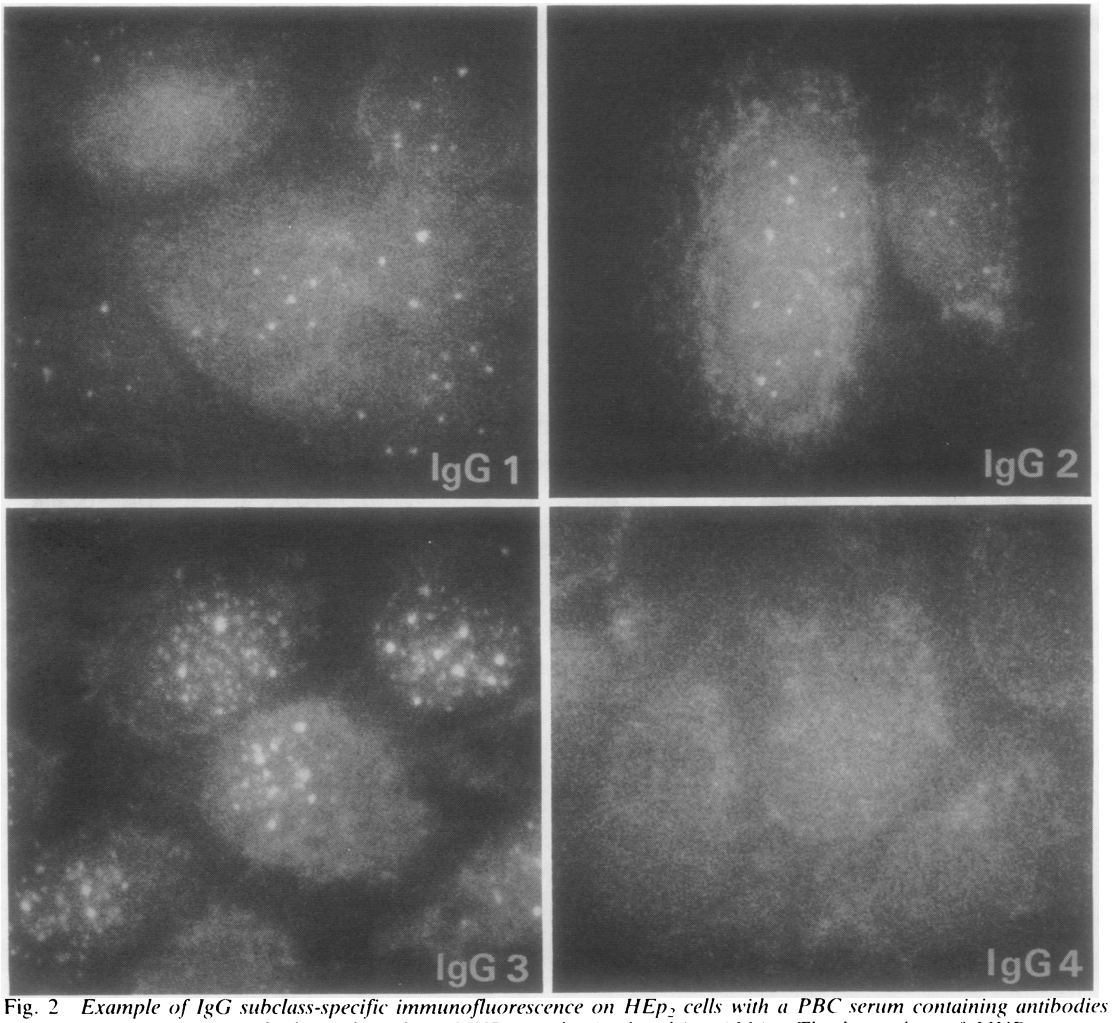

Fig. 2 *Example of IgG subclass-specific immunofluorescence on HEp_2 cells with a PBC serum containing antibodies to centromeres (ACA), multiple nuclear dots (MND), and mitochondria (AMA). The large dots of MND are clearly visible in the panels labelled IgG1, IgG2, and IgG3, whereas the smaller speckles of ACA are seen only with IgG3. The cytoplasmic staining of AMA is not so clear at these exposures but was restricted to IgG2 and IgG3. The IgG4 panel shows only background fluorescence after a long photographic exposure.*

Results

The frequency profiles of subclass reactivity are shown in Fig. 1. Anticentromere antibody was generally IgG1 and IgG3 (a mixture in 68% of cases and one or other in the remainder); only in one of the 50 cases was there any IgG4 staining, and IgG2 staining was never seen. Multiple nuclear dots (MND) staining was recognised as speckles rather larger in size than the centromeres, usually eight to 12 in number per interphase nucleus but absent from mitotic cells. In the nine cases studied (including seven also ACA positive) this antibody involved IgG subclasses 1, 2, and 3 but never 4.

Sixteen of the 50 ACA positive sera also gave rather weak nucleolar staining of the homogeneous type: this was varied in IgG subclass, often only involving one subclass, and most frequently IgG4. Antimitochondrial antibody was usually IgG3 and often also IgG2; IgG3 staining was brighter than IgG2 in most cases but not all. Sera containing anti-Scl-70 antibody gave the diffusely grainy pattern of nuclear immunofluorescence in all cases; this was IgG1 in all eight cases together with IgG3 in three cases and IgG4 in one. The speckled ANA pattern associated with anti-(U1)RNP antibody consisted of IgG1 and IgG4 in all cases, IgG3 in most cases, and IgG2 in only a few.

USE OF IgG SUBCLASS-SPECIFIC IMMUNOFLUORESCENCE TO ANALYSE COMPLEX AUTOANTIBODY PATTERNS

The restricted representation of IgG subclass profiles facilitated the recognition of one autoantibody in isolation from others in the same serum. Nucleolar staining was separated from the centromere pattern by IgG4 immunofluorescence, while antimitochondrial antibody and antibody to multiple nuclear dots were detected in isolation from ACA by the IgG2 reagent (Fig. 2). In this way, we found MND antibody in seven ACA positive sera: three (30%) of the 10 ACA positive PBC sera and four (10%) of the 40 sera from patients with CREST syndrome. Among the seven patients with both MND and anticentromere antibodies, the three with PBC had features of CREST syndrome, whereas the four cases diagnosed as CREST syndrome had no evidence of PBC (normal liver function and negative antimitochondrial antibody).

Discussion

The data presented here are no more than semi-quantitative but do clearly demonstrate restrictions of IgG subclass representation that differ from one autoantibody to another. Our findings on AMA agree substantially with those of Riggione *et al*,[24] but our results for anti-RNP differ from previous studies that were themselves discordant.[8 10] IgG1 and IgG3 were prominent in three of the six specificities studied (ACA, MND, and RNP), with IgG2 also frequent in MND and IgG4 in RNP antibody. On the other hand, Scl-70 antibody was predominantly just IgG1, AMA was chiefly IgG3 and IgG2, and antinucleolar antibody mainly IgG4.

These restrictions hold true whatever the underlying diagnosis. For instance, ACA was IgG1 and IgG3 whether the patient had CREST syndrome alone or with PBC, and the RNP antibody profile was similar whether the patient had scleroderma, mixed connective tissue disease, or SLE. On the other hand, IgG2 was particularly frequent in AMA and MND. These two antibodies are relatively specific for PBC, a disease also characterised by high serum levels of IgM,[25 26] and it is interesting to note that both IgG2 and persistent IgM antibody production are typical of immune responses independent of T cells, such as the B cell response to polysaccharide antigens[27]

The usual predominance of IgG1 and IgG3 subclasses found in this study extends also to anti-DNA and anti-Sm antibodies.[10] IgG1 and IgG3 are typical of immune responses dependent on T cells,[27] an inference consistent with the idea that autoantibodies may arise as antihapten responses when T cell tolerance to the carrier breaks down.[28 29]

The prominence of at least one of the IgG1 and IgG3 subclasses in all but the weak antinucleolar specificity argues against the hypothesis that led to this study. IgG1 and IgG3 have similar biological properties, including complement fixation and binding to cellular Fc receptors, that differ from IgG2 and IgG4.[30] It is unlikely, therefore, that associations between antibodies and disease can be explained by differences in biological function related to their subclass distribution.

There is a practical aspect to this study in the analysis of complex immunofluorescence patterns. For example, we found that antibody to multiple nuclear dots is often masked by the similar speckled appearances of anticentromere antibody, not only in primary biliary cirrhosis but also in patients with the CREST syndrome alone. The MND antibody frequencies reported here in ACA positive sera are 30% in PBC and 10% in CREST syndrome. These rates are considerably higher than the 13% and 0% reported previously in the 135 PBC sera and 161 scleroderma sera from which the material for the present study was in large part derived ($p<0{\cdot}05$, χ^2 analysis). Although features of the CREST syndrome affect 20% of patients with PBC (and at least half of these cases are ACA positive), only a small

proportion of patients starting out with CREST syndrome go on to PBC later. It will be of interest if antibody to multiple nuclear dots is shown to be a predictor of this uncommon, but usually fatal, outcome.

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