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Organ-specific IgM autoantibodies to liver, heart and brain in man: generalized occurrence and possible functional significance in normal individuals, and studies in patients with multiple sclerosis

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SUMMARY

In this paper we use a sensitive, ^{125}I-anti-immunoglobulin-binding assay, recently adapted for use with tissue homogenates as targets, to demonstrate autoantibodies to brain, liver and heart in the sera of normal persons. Quantitative absorption analyses demonstrated that the autoantigens detected were in each case organ-specific, and the brain autoantigen was shown to be present in equal amounts on cerebral cortex, cerebral white matter and cerebellar cortex. The autoantibodies were shown to be IgM in nature by gel filtration studies and experiments where IgM was reduced to monomers, and were found to bind equally well at 4, 20 and 37°C. Cross-reactions with brain, liver and heart of rat and dog were unpredictable and usually weak. Parallel studies with kidney homogenates failed to detect anti-kidney autoantibodies, but immunofluorescence studies on frozen sections revealed large amounts of immunoglobulin in normal kidneys, mainly on glomerular and tubular basement membranes, raising the possibility that autoantibodies to kidney are present but that the autoantigen sites are saturated *in vivo*. Sera from patients with multiple sclerosis were indistinguishable from normal sera in their binding to brain homogenate, and CSF from five patients with multiple sclerosis did not bind at all to brain homogenate. The theoretical and practical significance of multiple IgM autoantibodies in normal persons, and the organ specificity of the autoantibodies, is discussed.

INTRODUCTION

The mechanisms whereby the immune system distinguishes self from non-self are not known, but one clear area where there is unexpected reactivity against self-components is in the B lymphocyte system. The presence of autoantibodies, usually IgM in nature, in the sera of apparently normal individuals has been documented many times in both man and experimental animals (for review see Elson, Naysmith & Taylor, 1979). The proportion of individuals showing reactivity against any particular autoantigen has generally been small, of the order of 2 or 3%, and even where a large number of different autoantibodies have been sought in each individual, the cumulative total of individuals showing any autoantibodies has usually been less than 20% (e.g. Bullock, Booth & Wilson, 1979). Most studies, for reasons of experimental precision and convenience, have been done with leucocytes and erythrocytes. Studies on tissues not readily dissociable into single-cell

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suspensions have been done either by immunofluorescence, the sensitivity of which is unlikely to be such as to detect low levels of autoantibody, or else by less direct techniques such as complement fixation (e.g. Boss, Silber & Nelken, 1967).

An important technical problem in the search for autoantibodies, *particularly if they are almost always present*, is to distinguish assay 'backgrounds' due to non-specific adsorbance of immunoglobulin, from antigen-specific interactions of antibodies with tissues. A recent communication from this laboratory described an anti-immunoglobulin, radioactive binding assay using tissue homogenates as targets, and the potential advantages of this assay system for the detection of autoantibodies were discussed (Hart & Fabre, 1980). On screening normal, control sera on brain, liver and heart homogenate targets with this assay we were intrigued to find that the shape of the titration curves in these assay systems resembled in every respect that of an antiserum. As virtually every normal serum we examined behaved in this way, the only approach we could conceive that would distinguish convincingly between non-specific background binding of immunoglobulins and antigen-specific binding of autoantibodies, was to examine the tissue distribution of the putative target autoantigens by quantitative absorption analysis. What we found was a remarkable organ specificity in the binding of the putative autoantibodies to heart, liver and brain homogenate targets. This is difficult to explain on the basis of non-specific adsorbance of immunoglobulin or immune complexes to the homogenates and is most readily interpreted as being due to organ-specific autoantibodies. Virtually every normal serum we examined had autoantibodies against all three tissues, demonstrating a multiplicity of autoantibodies in each individual.

MATERIALS AND METHODS

Sera and cerebrospinal fluid. Normal human sera were obtained as follows: 12 from normal, healthy volunteers aged 20–35 years (seven male and five female); four from surgical patients (all female) aged 65, 76, 81 and 84 years, with no neurological or other known systemic illness; and four from children (two male and two female) aged 2–6 years, all in-patients with no neurological or other systemic illness. Blood from 10 patients and cerebrospinal fluid (CSF) from five patients with multiple sclerosis (age 19–59 years with median of 35 years; nine male, six female) were from patients under the care of Professor W. B. Matthews, Department of Neurology, Oxford University. All sera were heated at 56°C for 30 min prior to use. In some experiments, sera were mixed with an equal volume of 0·01 M dithiothreitol and incubated for 30 min at 37°C to reduce IgM to monomeric form, as described by Olson *et al.* (1976).

The rabbit anti-human $F(ab')_2$ antibody (RAH) used in the binding assays and immunofluorescence studies was immunoadsorbent-purified on human $F(ab')_2$ columns. The eluted antibody was pepsin-degraded to $F(ab')_2$ and then filtered on Sephadex G-200 (Pharmacia, Uppsala, Sweden), the $F(ab')_2$ peak of the antibody being pooled, as previously described in detail (Dalchau & Fabre, 1979).

Homogenates. Liver, kidney, spleen and heart were obtained fresh from cadaver kidney donors, and a piece of cerebrum was obtained fresh from the neurosurgery unit. The subregions of brain were obtained from a post-mortem performed within 2 hr of death. Brain, heart and liver from inbred rats and mongrel dogs were obtained from freshly exsanguinated animals.

Homogenates were prepared as described previously (Dalchau & Fabre, 1979; Hart & Fabre, 1980). The protein content of the homogenates was estimated by a modification of the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard, and was generally in the range of 20–35 mg/ml.

Erythrocyte (RBC) suspensions. Relatively pure RBC suspensions were obtained by three sequential centrifugations with removal of buffy coat and upper red cell layer each time. RBC were in 0·5% BSA in phosphate-buffered saline (PBS), and were counted on a Coulter counter model DN.

Binding assay. This was a modification of the assay described by Morris & Williams (1975), adapted for use with tissue homogenate targets and previously described in detail (Hart & Fabre,

1980). Briefly, 25 μl of the serum to be analysed was incubated for 1 hr on ice with 25 μl of the appropriate homogenate suspension at approximately 2 mg of homogenate protein/ml. The target homogenate was then washed twice, and incubated for a further 1 hr on ice with ^{125}I-RAH. After a further two washes the homogenate-bound radioactivity was measured in a gamma counter.

Absorption analysis. This has previously been described in detail (Dalchau & Fabre, 1979; Hart & Fabre, 1980). Briefly, sera were first titrated to define the dilution which represented conditions of target antigen excess in the assay system. Absorptions were then performed at this serum dilution by incubating equal volumes (80 μl) of the serum with tripling dilutions of the homogenate or cell suspensions for 1 hr on ice, followed by removal of the absorbing tissue by centrifugation.

Gel filtration. An upward flowing K16/100 column packed with Sephadex G-200 (Pharmacia, Uppsala, Sweden) was used. Sera to be chromatographed were heat-inactivated as above, and dialysed against 0·15 M NaCl, 0·01 M Tris, 0·02% NaN_3, pH 7·4, at 5°C, which was the eluting buffer. Flow rates were approximately 10 ml/hr, and 2-ml fractions were collected. Absorbance at 280 nm was recorded continuously using a Uvicord II (LKB Instruments, Sweden).

Fluorescence studies on frozen sections. Cryostat sections of 6 μm were cut from tissues obtained fresh and stored in liquid nitrogen before use. The sections were air-dried for 15 min at 4°C, and all subsequent procedures were carried out at room temperature. Sections were washed three times by incubating for 10 min in PBS. Test sera were added to the sections for 30 min, and the sections washed three times as above. Fluorescein-labelled RAH at 25 μg/ml was then added for 30 min, and after a further three washes, the sections were mounted in 90% v/v of glycerol in PBS and examined with a Leitz Ortholux II microscope. In control sections, RAH at 25 μg/ml was incubated with human $F(ab')_2$ at 250 μg/ml for 1 hr prior to use, to specifically block the fluorescein-labelled antibody.

RESULTS

Initial screening for anti-brain antibodies in patients with multiple sclerosis

The original aim of this study was to examine the sera of patients with multiple sclerosis for anti-brain autoantibodies, with a view to studying the tissue distribution and biochemical characteristics of the target autoantigen(s), since the assay system used in these experiments readily lends itself to this sort of work. In the initial experiments, sera from five normal individuals and five patients with multiple sclerosis were titrated with brain homogenate targets, and the results are given in Fig. 1. It can be seen that there is no difference in the binding to brain homogenate in the two groups of sera. The striking feature of the titration curves, however, is that they resemble in every respect that of an antiserum. Thus, while it is clear that the sera of multiple sclerosis patients bind to brain homogenate in the same way as normal sera, should this binding be dismissed as 'non-specific background'?

We examined for binding to brain homogenate the sera from another five patients with multiple sclerosis, another seven healthy young adults, and sera from four children aged 2–6 years and four elderly persons aged 65–84 years. All except one of the sera from the young adults gave precisely the same picture as in Fig. 1. The unusual serum gave low binding, with a plateau around 7,000 c.p.m. up to a dilution of 1/27.

CSF from five patients with multiple sclerosis was examined and none of these bound to brain homogenate. Three of these CSFs had raised IgG/albumin ratios (32, 61 and 82%), the IgG content being 6·6, 20·9 and 23 mg/dl respectively. All five CSFs gave very similar titration curves, and the results of two of these are given in Fig. 1.

Binding of normal sera to brain, liver, heart and kidney homogenates

The high binding of virtually every serum we examined to brain homogenate raised the question of the sort of binding we would see with other homogenates. Sera from five healthy young adults were each examined for binding to brain, liver, heart and kidney homogenate targets. All five gave the same picture, and the results with three of the sera are given in Fig. 2. It can be seen that the titration curves with liver and heart homogenate resembled that seen with brain homogenate. The curves with kidney homogenate were substantially less steep.

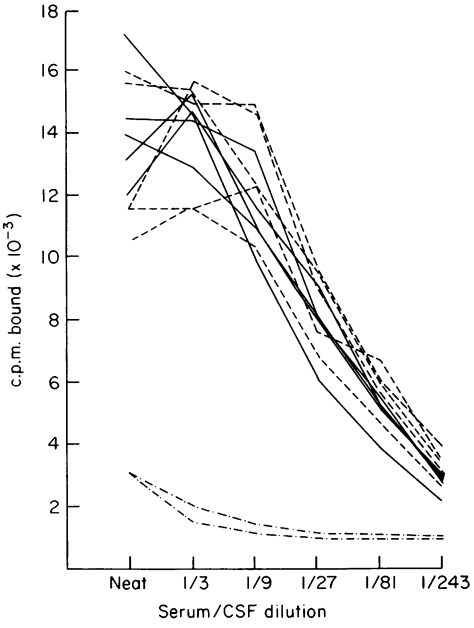

Fig. 1. Binding to brain homogenate targets of normal sera (——), and of sera (– – – –) and CSF (– · – · – ·) from patients with multiple sclerosis. Each line represents a different individual. c.p.m. bound refers to ^{125}I-labelled immunoadsorbent-purified rabbit F(ab')$_2$ anti-human F(ab')$_2$ (RAH).

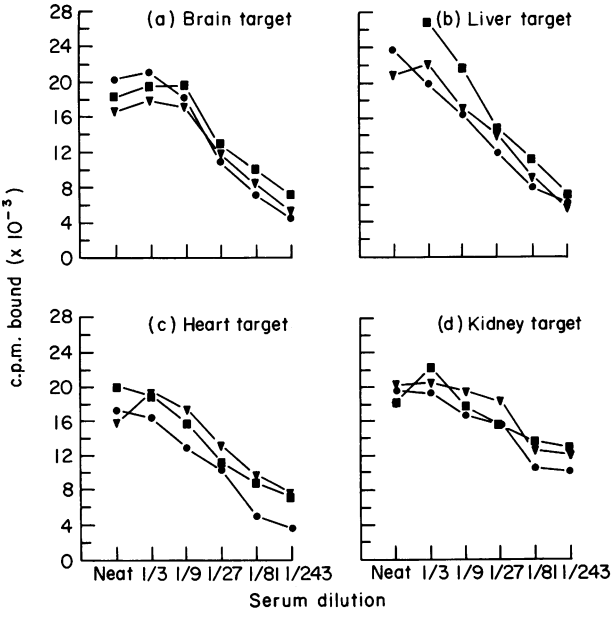

Fig. 2. Binding of sera from three normal persons to homogenates of (a) brain, (b) liver, (c) heart, and (d) kidney. The same three sera were used on the four homogenate targets. c.p.m. bound as in Fig. 1.

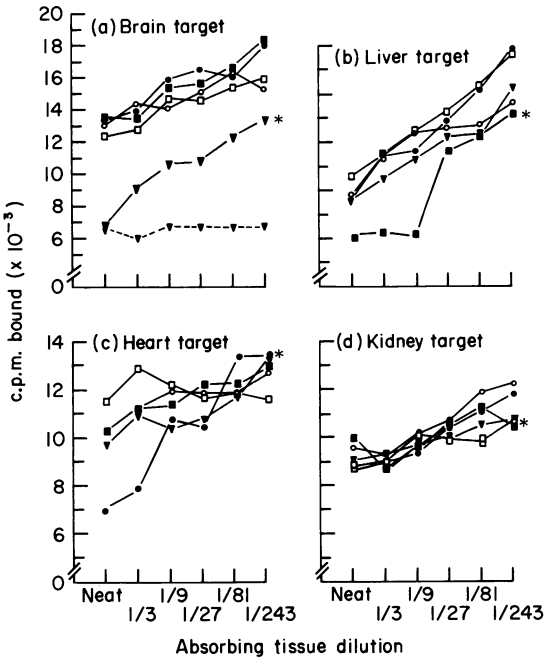

Fig. 3. Quantitative absorption analysis of a normal serum using assays with (a) brain, (b) liver, (c) heart, and (d) kidney homogenate targets. In each assay system the serum was absorbed at a 1/5 dilution with tripling dilutions of homogenates of brain (▾), liver (■), heart (●), kidney (□), and spleen (○). In (a) (▾- - -▾) exhaustive absorption as described in Materials and Methods where the serum was preabsorbed with the pellet after centrifugation (to avoid serum dilution) of an equal volume of brain homogenate prior to the quantitative absorption shown. * Absorbing tissue the same as the target tissue. c.p.m. bound as in Fig. 1.

Tissue distribution of putative autoantigen recognized in the assays with different homogenate targets
Two sera from healthy young adults were each absorbed quantitatively with brain, liver, heart, kidney and spleen homogenates, and assayed back on brain, liver, heart and kidney homogenates. Both sera gave the same picture, and the results with one of the sera are given in Fig. 3. We were surprised to find a remarkable organ specificity as regards the putative autoantigens detected by the assays on brain, liver and heart. Thus Fig. 3a shows that with brain homogenate targets only brain gave good absorption, the other tissues giving slight and probably non-specific absorption, which in any case amounted to only 1–2% of the absorptive capacity of brain. The picture using heart homogenate targets (Fig. 3c) was similar, in that only heart tissue gave good absorption. The results with liver homogenate targets (Fig 3b) were slightly more complex in that although liver clearly had 10–20-fold more of the putative autoantigens than brain, heart, spleen and kidney, the absorption curves with these tissues suggested that the autoantigen was present on these tissues, although in much reduced quantity. With kidney homogenate targets none of the tissues gave any significant absorption, not even kidney homogenate itself, which is the sort of picture one would expect if there were no specific binding of the serum.

The above data is most readily interpreted on the basis that we are dealing with brain-, liver- and heart-specific autoantibodies in the three assay systems. The homogenates clearly control very nicely for one another in each system.

In addition to the complete absorption studies done on the two sera as discussed above, a further four sera from normal healthy young adults and three sera from patients with multiple sclerosis were studied using quantitative absorptions with brain and liver homogenates in an assay system with brain homogenate targets. All seven sera showed excellent absorption by brain and negligible absorption by liver, similar to that seen in Fig. 3a.

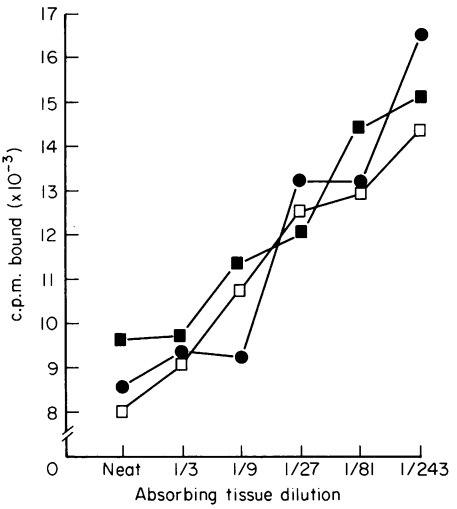

Fig. 4. Quantitative absorption analysis of a normal serum at 1/3 dilution with homogenates of cerebral cortical grey matter (●), cerebral white matter (□) and cerebellar cortex (■). Brain homogenate was used as the target in the assay system. c.p.m. bound as in Fig. 1.

Presence of the brain autoantigen on different subregions of the brain

Quantitative absorption analyses with three of the normal sera were performed using cerebral cortical grey matter, cerebral white matter and cerebellar cortex and then assayed back on brain homogenate. The results with all three sera were virtually identical, and the results with one of the sera are given in Fig. 4. It is clear that the brain autoantigen is present in approximately equal amounts in all three regions of the brain. This makes it unlikely that the autoantibody is directed against a myelin-specific component.

Absence of temperature dependence of binding.

As many autoantibodies bind preferentially or exclusively in the cold, and as our assays are routinely performed at 4°C or on ice, we tested the binding of one of the sera to brain, liver and heart homogenate at various temperatures. The incubation of homogenate and sera, plus the two washes following this incubation, were performed at 4, 20 and 37°C. The rest of the assay was performed at 0–4°C as usual. With all three homogenates, the binding of the serum was much the same at all three temperatures (data not shown). This binding at physiological temperatures increases the possibility that the autoantibodies might have a physiological function *in vivo*.

Exclusion of binding to tissue homogenates of antibodies to the ABO system

The precise tissue distribution of the antigens of the ABO system are not known, but in any case with a new assay system it is possible that previously undetected amounts of antigen might be a problem. We therefore did quantitative absorptions with A and B rhesus-positive erythrocytes on three normal sera each assayed on brain, liver and heart homogenates. In none of the nine analyses did the erythrocytes give any absorption, while the specific homogenate gave the expected absorption pattern.

Class of the autoantibodies

Two series of experiments were performed to establish the class of the brain-, liver- and heart-specific autoantibodies.

In the first experiment, normal serum was chromatographed on a Sephadex G-200 gel filtration column, and the fractions were assayed for binding to brain, liver and heart homogenate. The results with the three homogenates were virtually identical, and the data for the assays on brain homogenate are given in Fig. 5. Virtually all the binding was associated with the void volume of the column, which strongly suggests that the autoantibody is IgM. There was a small binding peak of

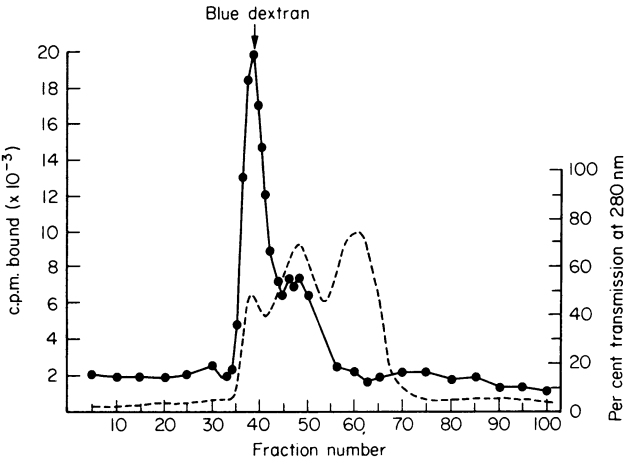

Fig. 5. Fractionation of a normal serum by Sephadex G-200 gel filtration. Each fraction was tested for binding to brain homogenate targets (•), and absorbance at 280 nm (- - - -) was monitored continuously. Blue dextran indicates the void volume of the column. c.p.m. bound as in Fig. 1.

6,000–7,000 c.p.m. corresponding to the IgG fraction, but if one remembers that the true assay background in the presence of serum (established by exhaustive brain absorption of the serum in Fig. 3a) is also 6,000–7,000 c.p.m., it appears likely that this second peak represents non-specific binding of immunoglobulin to the homogenate.

The second experiment involved incubating the sera with dithiothreitol under conditions known to dissociate IgM into its monomeric form (Olson *et al.*, 1976). Sera from three normal persons were treated with dithiothreitol or with a control buffer and then tested for binding to brain, liver and heart homogenate. A further three normal sera were treated in the same way but tested for binding only to brain homogenate target. In all cases, the sera treated with dithiothreitol showed much reduced binding, suggesting that at least most of the antibody was IgM and that reduction to the monomeric form substantially diminished the avidity of binding. This result is of interest also because it is inconsistent with the binding of the sera to the homogenates being due to immune complexes or aggregated IgG.

Studies with kidney

Our results with kidney were atypical in relation to those with brain, liver and heart in that the initial titration curve was shallow (Fig. 2d), there was no absorption by kidney or other tissues in the assay with kidney homogenate target (Fig. 3d) and backgrounds were very high. To investigate this problem we took normal kidneys, and examined cryostat sections directly for human immunoglobulin by staining with fluorescein-labelled RAH, with human $F(ab')_2$-blocked fluorescein-labelled RAH as the control. The results are given in Fig. 6. The most brightly staining structure in the kidney was the glomerular basement membrane (Fig. 6a). Occasional clusters of tubules also showed substantial staining, which appeared to be throughout the cell cytoplasm (Fig. 6c). Most tubules in both cortex (Fig.6c) and medulla (Fig. 6d) showed basement membrane staining with some staining in the interstitial connective tissue. In the medulla certain areas stained more brightly than others (Fig. 6d). Control sections in all areas were almost completely black (e.g. Fig. 6b).

In the context of our preceding results with brain, liver and heart, it is possible that the immunoglobulin seen in kidney represents autoantibody binding to its target antigen. If this is so and the autoantigen sites are at or near saturation, this would explain the high assay backgrounds and the shallow titration curve with kidney homogenate target (Fig. 2d) and the failure of kidney to absorb activity in the absorption analysis with kidney homogenate targets (Fig. 3d). This is not a point we would wish to labour, but the possibility that the immunoglobulin seen in normal kidney is autoantibody should at least be considered.

Cryostat sections of brain and liver were also examined for the presence of immunoglobulin as detailed above for kidney, but very little was present except in the immediate vicinity of blood

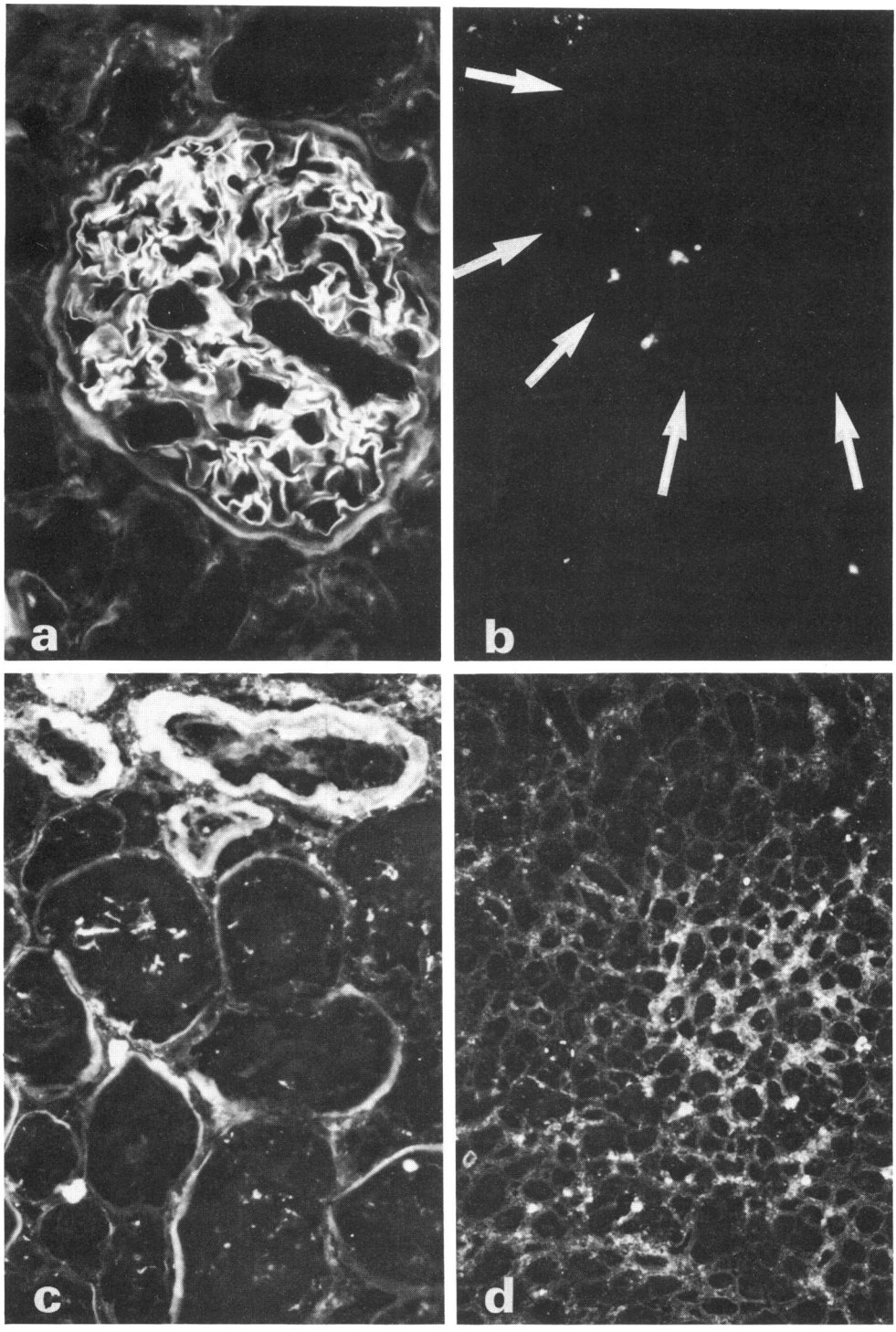

Fig. 6. Immunofluorescence on frozen sections of human kidney. To detect the presence of immunoglobulin in normal kidney, sections were incubated with fluorescein-labelled, immunoadsorbent-purified rabbit $F(ab')_2$ anti-human $F(ab')_2$ (RAH) at saturating concentrations (25 μg/ml) (a, c & d). The control section (b) was incubated with fluorescein-labelled RAH blocked by preincubation before addition to the section with a 10-fold molar excess of human $F(ab')_2$. (a) Glomerulus. ($\times$ 200.) (b) Control section. Outline of a glomerulus is marked by arrows ($\times$ 200). (c) Renal cortex. ($\times$ 200.) (d) Renal medulla. ($\times$ 80.)

vessels. There was virtually no staining of brain, and in liver there was a very occasional bright cluster of cells, which we were unable to identify, and faint staining of bile canaliculi.

Attempts to localize the autoantigens on frozen sections

The IgM fraction of one of the sera was absorbed with either liver or brain homogenate and tested on frozen sections of liver and cerebral cortex. For each frozen section, the antibody absorbed with the specific tissue served as the negative control, while antibody absorbed with the other tissue served as the test. However, with neither liver nor brain could we convincingly detect specific binding, even though the backgrounds were good and the conditions for fluorescence optimal. There was a suggestion of binding to neuronal membranes in the cerebral cortex, but this was too weak for definitive comments.

Species specificity of the autoantibodies

The anti-brain autoantibody was well absorbed by dog brain but only weakly absorbed by rat brain, which absorbed to a plateau after removing approximately half of the activity. Conversely, the anti-heart autoantibody was well absorbed by rat heart but poorly absorbed by dog heart. Both dog and rat liver homogenates gave relatively weak absorptions of the anti-liver autoantibody.

DISCUSSION

Our data suggest that virtually all normal individuals have multiple IgM autoantibodies. This is the simplest and most reasonable interpretation of our demonstration of organ-specific binding of immunoglobulin in normal sera. The possibility that we are dealing with immune complexes showing organ specificity in their binding properties is unlikely. Even if this were so, however, the organ specificity of the complex would have to be mediated by the antigen moiety of the complex, via some ligand/receptor interaction involving the antigen and the tissue in question. The major conclusion of this study, that multiple autoantibodies exist in virtually all normal persons, would therefore still hold, since different autoantigen/autoantibody complexes would be involved in the brain, liver and heart assay systems.

In no previous study has a virtually universal incidence of multiple autoantibodies been reported. This is perhaps because less sensitive techniques have been used, but, of most importance, even if other assay systems did detect binding with all normal sera, this is likely to have been ignored as the non-specific background of the assay. It is exceedingly difficult to prove that what is being observed is antigen-specific binding of autoantibodies, rather than non-specific binding of immunoglobulins, *when the same binding is seen with all normal sera.* The fact that our assay system was sufficiently versatile to allow a study of the tissue distribution of the putative autoantigen by quantitative absorption analysis, and the entirely unexpected observation that the autoantigens detected were organ-specific, enabled us to conclude that the observed binding was due to autoantibodies.

Two major questions arise. The first is the significance of naturally occurring autoantibodies, especially if they are found universally and are directed at many self-components. It has been suggested that autoantibodies have a physiological role not directly connected with immunity, i.e. that they might function to assist the phagocytosis of dead or dying cells by binding to antigenic sites not exposed on healthy cells (Grabar, 1975). However, we would favour an explanation more directly connected with the physiology of the immune system. It has been suggested that autoantibodies might be involved in self-tolerance, by various blocking mechanisms (Hellström & Hellström, 1972). While blocking mechanisms would seem unlikely to be involved in self-tolerance, we feel that a role of the autoantibodies in this area is an intriguing possibility. Jerne's theory of a network of idiotypes and anti-idiotypes (Jerne, 1974) and the finding that suppressor T cell mechanisms involve two subsets of suppressor T cells, one with anti-idiotype specificity (Weinberger *et al.*, 1980), raise the possibility that the autoantibodies might be involved in maintaining a network of anti-idiotype-specific suppressor T cells. It is of interest that patients with agammaglobulinaemia have an increased incidence of autoimmune disorders (Fudenberg, 1971),

but whether or not this can be related to an involvement of autoantibodies in self-tolerance must wait upon a better understanding of the pathophysiology of agammaglobulinaemia.

It is also possible that the autoantibodies are an innocuous and trivial by-product of the fact that self-tolerance might be concerned primarily with the T cell system (Allison, 1971). The IgM autoantibodies might represent the activity of B cells reacting without T cell help with autoantigens with which they happen to come into contact. In any case, whether or not there is any relationship between this physiological autoreactivity and pathological states of autoimmunity is entirely unknown.

The second important question is why the autoantibodies should show such organ specificity. This was a fortunate and welcome but unexpected result. It could be related to the possibility that the major membrane glycoproteins of different tissues might be differentiation antigens which are tissue-specific or at least have a restricted tissue distribution. This is certainly the case with the lymphocyte membrane glycoproteins of the rat (Standring *et al.*, 1978) and appears also to be the case with the human lymphocyte membrane (Dalchau, Kirkley & Fabre, 1980a, 1980b). That the surface antigens of other tissues might show the same high degree of tissue specificity seen with lymphocytes is suggested by the finding that, in the rat, autoantibodies induced by immunization with rat liver (Hart & Fabre, 1981a) and rat kidney (Hart & Fabre, 1980)—and to a slightly lesser extent with rat heart (Hart & Fabre, 1981b)—show a high degree of organ specificity.

In our studies in patients with multiple sclerosis we could not detect any increase over normal in anti-brain autoantibodies. While this does not exclude the possibility that patients with multiple sclerosis have anti-brain autoantibodies not seen in normal persons, they clearly indicate that great care is necessary before such autoantibodies are designated as distinctively present in multiple sclerosis. This area is a controversial one (Fraser, 1975), although antibodies to oligodendrocytes appear to be present in the serum of patients with multiple sclerosis (Abramsky *et al.*, 1977). One clear point, however, is that none of the five CSFs from patients with multiple sclerosis, three of which had elevated levels of IgG, had anti-brain autoantibodies. This suggests that the specificity of the abnormal IgG in the CSF of patients with multiple sclerosis is not directed at brain autoantigens.

The final point we wish to make is in relation to the species specificity of the autoantibodies. It is frequently the practice in searching for human autoantibodies by immunofluorescence to use tissue from other species, often as phylogenetically distant as the rat. In such studies one is relying on interspecies cross-reactivity of the autoantibody and our studies clearly show (see last section of Results) that it is unwise to depend on such cross-reactions.

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