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Characterization and Pathological Significance of Monoclonal DNA-Binding Antibodies from Mice with Experimental Malaria Infection

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Malaria infection is accompanied by the production of a number of autoantibodies, including some that react with DNA. Epidemiological evidence implicates these in the nephritides that arise in human quartan malaria and in experimental malaria infections in mice. Through parallels with the involvement of DNA-reactive antibodies in the autoimmune syndrome systemic lupus erythematosus, a role for DNA-reactive antibodies in forming phlogistic immune deposits in the kidneys is implied. To more fully understand the relationship between antibodies of this specificity made in malaria and systemic lupus erythematosus, we prepared monoclonal DNA-reactive antibodies from BALB/c mice infected with *Plasmodium berghei* (clone RC) and compared their properties with those of other antibodies previously isolated from lupous MRL/*Mp lpr/lpr* and (NZB × NZW)F_1 mice. Antibodies from malarial mice were all immunoglobulin M class and bound to single-stranded but not double-stranded DNA in an enzyme-linked immunosorbent assay. They also reacted with synthetic polyribonucleotides in the enzyme-linked immunosorbent assay and with parasitized erythrocytes and parasite pigment in kidney sections. None of the antibodies from lupous mice had identical specificities. The potential involvement of the DNA-reactive antibodies in malarial nephritis was demonstrated, by use of immunocytochemical methods, on the basis of their binding to existing immune deposits in kidney sections from malarial mice, a similar property having been previously demonstrated for antibodies from lupous mice. Furthermore, antibodies from malarial mice expressed public idiotypes, notably Id.V-88, which is a member of the Id.16/6 family, commonly found on DNA-reactive antibodies in lupus and other infectious and connective tissue diseases. This study indicates that DNA-reactive antibodies in malaria have immunochemical properties similar but not identical to those of such antibodies in systemic lupus erythematosus and that they have the potential to participate in the formation of immune deposits in nephritic malarial kidneys.

Increased levels of antibodies that bind DNA are a recognized characteristic of some autoimmune diseases, notably systemic lupus erythematosus (SLE). Although such antibodies are sometimes present in healthy individuals (26), a good correlation has been found between disease activity and high titers of DNA-binding antibodies in SLE (29). Particular attention has focused on their potential role in the development of kidney disease during SLE. A strong etiological association has been found between DNA-binding antibodies and SLE-associated nephritis, and studies have shown that increased titers of these antibodies correlate with the onset or development of nephritis (2).

DNA-binding antibodies have also been found in the sera of patients with a variety of parasitic infections, such as malaria, leprosy, or schistosomiasis (1, 15, 24, 30, 34). Malaria infections, in particular, may be associated with renal complications, and it has been suggested that DNA-binding antibodies may contribute to this pathology (1, 12). We previously studied the contribution of DNA-binding antibodies to the initiation and development of the nephritis that occurs during murine malaria (31) and found that increased levels of these antibodies occurred during the middle and late stages of infection and that they may well be involved in the exacerbation of renal injury occurring during the early stages of infection, particularly during chronic murine malaria. The purpose of the present study was to compare more closely the properties of the DNA-binding antibodies made in malaria and in SLE. To this end, we prepared monoclonal antibodies (MAbs) from mice with *Plasmodium berghei* infection and compared them with DNA-binding antibodies derived from mice that developed SLE (20) to examine their potential role in the concomitant kidney pathology observed in malaria infections.

MATERIALS AND METHODS

Parasite maintenance. Infection with the chronic malaria parasite *P. berghei* (clone RC) was initiated and maintained by blood passage: 5×10^3 infected erythrocytes were injected intraperitoneally into 6-week-old female LACA or BALB/c mice (Tuck & Sons, Essex, England), and the course of infection was monitored by daily examination of blood samples taken from the tail. Smears were fixed in methanol and stained with Giemsa stain, and percentages of parasitized erythrocytes were calculated by examining 2,000-cell samples in random fields.

MAbs. Female BALB/c mice, 8 to 10 weeks of age, were infected with *P. berghei* (RC). By 14 to 15 days after infection, the mice had elevated levels of circulating antibodies reactive with DNA; at this stage, the animals were killed and their

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spleen cells were used to prepare MAbs by standard methods (18, 20). Spleen cells were fused with approximately 1/10 their number of Sp2/0 myeloma cells. Microcultures were established in flat-bottomed multiwell plates (2 ml per well), and hybridomas were selected in hypoxanthine-aminopterin-thymidine medium. Stable cell lines were isolated by at least three cycles of cloning at limiting dilution. The isotypes of the MAbs were determined by double diffusion in an agarose gel with specific anti-isotype reagents (Sigma, Poole, Dorset, England). The MAbs described here are all of the immunoglobulin M (IgM) isotype and were generated from three individual fusions: MAb MA-1 was derived from fusion 1207, MAbs MA-2 and MA-3 were derived from fusion 1214A, and MAbs MA-4, MA-5, and MA-6 were derived from fusion 1214B.

MAbs derived from (NZB × NZW)F_1 (BWF1) and MRL/*Mp lpr/lpr* (MRL/lpr) mice were prepared from nonimmunized lupous mice and characterized as described before (20, 23, 27). The antibodies are I-410 and I-402 (specific for double-stranded DNA [dsDNA]), II-212 and III-152 (reactive with both dsDNA and single-stranded DNA [ssDNA]), IV-228 (specific for ssDNA), and V-88 and F-423 (reactive with ssDNA and also with RNA and dsDNA, but only to a small extent).

ELISA for anti-DNA activity. Serum antibodies and MAbs, as culture supernatants, were titrated in a direct enzyme-linked immunosorbent assay (ELISA) against ssDNA from calf thymus (Sigma) by the method established in our laboratory (20, 23). The only significant modification was that casein was omitted from reagent and washing buffers. The ELISA was adapted for use as a competition assay in which the competing ligand was serially diluted in the presence of a limiting amount of a MAb reactive with ssDNA (20). The results are expressed as the interpolated concentration of ligand in the titration curve giving 50% inhibition of binding. Competitive ligands were ssDNA, dsDNA, and synthetic polynucleotides (Sigma) poly(I) [p(I)] (single-stranded polyribonucleotide), p(I · C) (double-stranded polyribonucleotide), p(dG-dC) · p(dG-dC) (double-stranded polydeoxyribonucleotide), and p(A · U · U) (triple-stranded polyribonucleotide).

Assay for Id expression. Purified rabbit antibodies raised against DNA-reactive MAbs I-402, IV-228, and F-423, derived from MRL/lpr mice, and MAb V-88, derived from a BWF1 mouse, were prepared and used in a two-site binding assay as described before (23, 27). In brief, anti-idiotype (Id) antibodies (e.g., anti-Id.402) were immobilized at fixed concentrations in microtiter plate wells, and the unknown serum or MAb was titrated by serial dilution. Capture of Id-positive immunoglobulin (Ig) was determined by a conventional ELISA with a peroxidase-labelled rabbit anti-mouse Ig reagent. A standard curve was generated for each reference MAb, which was ascribed an arbitrary Id activity of 10^4 units per ml. The inhibitory activities of the MAbs from malarious and lupous mice were related to each curve, and the specific activities were determined by relating these values to their anti-ssDNA titers in the direct DNA ELISA.

DNA-binding antibody attachment to kidney sections. Kidneys were removed from uninfected mice and mice during the later stages of infection with *P. berghei* (RC). The kidneys were mounted on cork platforms in OCT compound (BDH, Poole, England), snap frozen in liquid nitrogen, and stored at −80°C. Sections (4 μm) were prepared by cryotomy, placed on 12-well Multitest slides (Flow Laboratories, Rickmansworth, England), fixed for 20 min at 4°C in acetone, and stored dessicated overnight at room temperature. Sections were stained with a streptavidin-biotin staining system described previously (31). Three sequential blocking steps were used to prevent nonspecific binding; the sections were overlaid with avidin and then biotin (20 min for each step; Vectastain kit; Vector Laboratories, Peterborough, England) and then with 20% fetal calf serum in phosphate-buffered saline (PBS) for 15 min. The preparations were washed twice with PBS for 5 min between each step of the process. The sections were then overlaid with protein A-purified IV-228 or I-402 (diluted 1/10 in PBS [pH 7.4] containing 10% normal mouse serum and 1% bovine serum albumin) or neat tissue culture supernatant containing one of the MAbs in the MA-1 to MA-6 series. The sections were then overlaid with biotinylated rabbit anti-mouse Ig (Jackson Immunoresearch Laboratories, Stratech Scientific Ltd., Luton, England) and then streptavidin-peroxidase complex (Amersham Ltd., Aylesbury, England) (both for 30 min at a 1/100 dilution). Finally, the slides were flooded with peroxidase substrate solution (diaminobenzidine [400 μg/ml] in PBS containing 0.02% cobalt chloride, 0.015% nickel ammonium chloride, and 0.01% H_2O_2) and counterstained for 10 min in 5% Light Green in distilled water (BDH) before being examined microscopically.

Separation of parasitized erythrocytes. Parasitized erythrocytes were separated by density gradient centrifugation as described by Knight and Sinden (16). In brief, packed washed erythrocytes from mice infected with *P. berghei* (RC) were centrifuged on a discontinuous Percoll (Pharmacia, Milton Keynes, England) gradient at 650 × *g* for 15 min at 4°C. Five separate interfaces were obtained, and of these, the top one was discarded and the other four were washed in Tris-buffered saline before smears were made. These smears were fixed in methanol and stained with Giemsa stain to determine the percentage and form of the parasites present in each band. The proportions of trophozoites, schizonts, and uninfected erythrocytes were estimated. Concentrations of cells in each layer were adjusted to values suitable for microscopic analysis. Samples were added in 5-μl droplets to duplicate wells of 10-well Multitest slides (Flow), which were then air dried and acetone fixed for 10 min before being stored dessicated at −20°C.

Indirect immunofluorescence to show attachment of DNA-binding antibodies to separated parasitized erythrocytes. Slides were brought to room temperature under humidified conditions for 3 to 4 h and then were refixed in acetone at 4°C for 20 min. After thorough air drying at room temperature, the slides were rehydrated by being washed in PBS for 20 min. Each well was overlaid with 20 μl of MAb, the malaria MAbs being used neat and the lupus MAbs being used diluted 1/10 in 10% normal mouse serum in PBS. After incubation in a humidified chamber for 1 h, the slides were washed twice for 5 min each time in PBS. Each well was then overlaid with 20 μl of a 1/100 dilution of fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig (Nordic Immunochemicals Ltd., Maidenhead, England). After incubation at room temperature in the dark for 1 h and further washing in PBS, the wells were overlaid with Citifluor retardant (Citifluor Ltd., City University, London, England) and mounted under coverslips for microscopic examination.

RESULTS

Serological reactivity of MAbs from malarial mice with DNA. The six MAbs derived from malarial mice each had a distinctive antigen-binding profile. They all reacted with ssDNA in the direct binding ELISA but not with dsDNA. In doing this they resembled MAbs, such as IV-228, derived from a lupous MRL/lpr mouse, that are functionally specific for ssDNA. To determine more clearly the ligand-binding profiles

TABLE 1. Comparative antigen-binding properties of DNA-reactive MAbs from malarial mice, mice with SLE, and normal BALB/c mice[a]

Mice and MAb	Concn of the following inhibitor (μg/ml) required for 50% inhibition of MAb binding to ssDNA:					
	ssDNA	dsDNA	p(I)	p(I·C)	p(A·U·U)	Dextran SO_4
Malarial						
MA-1	5.7	630	81.3	>50	0.49	>1,000
MA-2	18.8	>1,000	85.6	26.9	>500	>1,000
MA-3	6.25	>1,000	75	30	>500	>1,000
MA-4	20.7	500	87.5	>50	>500	>1,000
MA-5	5	>1,000	<2.5	33.1	>500	>1,000
MA-6	12	>1,000	<2.5	0.6	>500	>1,000
MRL/lpr lupous						
I-410	50	44	>625	>50	>500	1,000
II-212	17.8	>1,000	487	>50	>500	>1,000
III-152	3.6	590	330	>50	>500	>1,000
IV-228	18	515	>625	>50	>500	>1,000
F-423	2.2	51	6.6	>50	>500	>1,000
Normal BALB/c						
N-454	91	43				
N-455	2	55				

[a] Data are from a representative experiment. Each test was repeated three or four times.

TABLE 2. Id expression by DNA-binding MAbs from mice with malaria

MAb	ssDNA binding (direct binding ELISA titer^{-1})	Expression (U/ml)[a] of:			
		Id.V-88	Id.I-402	Id.IV-228	Id.F-423
MA-1	250	600	60	48	196
MA-2	27	830	126	56	189
MA-3	66	300	71	63	455
MA-4	50	2,560	300	48	>1,000
MA-5	43	0	63	56	NT
MA-6	49	370	71	82	149

[a] Activities of individual MAb preparations measured in the Id sandwich ELISA. A_{450} values obtained for individual samples were related to a standard curve obtained from titrating the homologous MAb, such as MAb V-88, in the anti-Id.V-88 ELISA, and so on. Because Ids are measured in arbitrary units, the relative levels of expression of different Ids by one MAb cannot be directly compared. Data are from one of two experiments. Each datum point was derived from duplicate observations. NT, not tested.

of the antibodies, each was examined in a competition ELISA that allows a direct comparison of the amounts of different ligands required to achieve equivalent inhibition of antibody binding (Table 1). In each case, the relative specificity for ssDNA was confirmed, and only MA-1 and MA-4 had detectable, but weak, reactivity with dsDNA under the test conditions. In comparison, MAbs from lupous mice reacted with ssDNA and dsDNA according to their previously described patterns (20, 23). The MAbs from the malarial mice were also different in this regard from two IgM MAbs (N-454 and N-455) which were isolated independently from normal, nonimmunized BALB/c mice and which reacted with both dsDNA and ssDNA.

None of the MAbs listed in Table 1 reacted at all with RNA in the competition assay. However, the MAbs from the malarial mice all reacted, some very strongly, with polyribonucleotides, such as p(I), a property they shared with MAb F-423, isolated from a fetal MRL/lpr mouse. On the other hand, this property distinguished them from the MAbs isolated from adult MRL/lpr mice, which reacted only weakly with p(I). Four MAbs from malarial mice also reacted with double-stranded p(I·C), which was not an antigen recognized by the MRL/lpr MAbs. MAb MA-1 uniquely reacted very strongly with triple-stranded p(A·U·U). None of the MAbs reacted with the double-stranded synthetic deoxyribonucleotide p(dG-dC)·p(dG-dC), which can take up a configuration like Z-DNA, in a direct binding assay (data not shown).

From these reaction profiles, it is clear that the MAbs derived from the same mice are independent isolates (MA-1; MA-2 and MA-3; and MA-4, MA-5, and MA-6). Furthermore, the antibodies associated with the malaria infection studied here do not have reactivity profiles identical to those of antibodies from mice that have SLE.

Id expression by DNA-binding MAbs from malarial mice. In the Id sandwich ELISA, several of the MAbs were found to express Ids previously associated with DNA-reactive MAbs from lupous mice (Table 2). Thus, Id.V-88, which is a member of the Id.16/6 family (21, 28), was strongly expressed on MA-4 and, to a lesser extent, on MA-2 and MA-1. MA-4 also expressed Id.I-402, as did MA-2. Id.F-423, which is related to Id.V-88 (23, 27), was detected on MA-3 and MA-4 and less strongly on MA-1 and MA-2. The two MAbs (MA-2 and MA-4) most clearly positive for the Ids tested were derived from different individual mice, which themselves were strongly positive for the expression of Id.V-88 and Id.F-423 on their circulating serum Igs (data not shown) at the time of sacrifice. These mice were also positive for the other Ids that were less apparent or not detected on the MAbs derived from them. Because these public Ids are also expressed on some normal Igs (13), it is not realistic to set a level for negativity, but the different rankings of Id expression by the MAbs show that they have varied Id profiles.

Binding of DNA-reactive MAbs to kidneys of malaria-infected mice. DNA-reactive MAbs derived from malarial mice bound to kidney tissue from mice in the middle or late stage of infection, and each showed a staining pattern similar to that observed with the lupous mouse-derived MAb IV-228 (Fig. 1a). Staining with MAb MA-1, which was typical of the antibodies derived from the malarial mice, was primarily mesangial; few cell nuclei were stained (Fig. 1b). None of these MAbs reacted with cell nuclei (essentially composed of dsDNA), in accordance with their specificity for ssDNA and their lack of reactivity with dsDNA in the ELISA. There was no difference between the binding of different MAbs from malarial mice. In areas with a heavy interstitial infiltrate, the phagocytosed parasite pigment was also stained (Fig. 1c), implying that the MAbs recognized parasite antigens as well as DNA. Antibody attachment was not observed in any of the control preparations or in sections from uninfected mice (Fig. 1d) or mice in the early stage of infection.

Binding of DNA-reactive antibodies to parasitized erythrocytes. A distinct separation between life cycle stages in erythrocytes recovered from the different gradient fractions was not obtained. Thus, we chose to compare the binding of different MAbs to cells from fraction 2, because this fraction contained parasitized erythrocytes (68.5% trophozoites and 31.5% schizonts) but no uninfected erythrocytes. The staining patterns elicited by the malarial and lupous populations of DNA-binding antibodies differed. Two main staining patterns were observed on parasitized erythrocytes: one of discrete spotty pinpricks and the other more diffuse. The spotty pattern was characteristic of the lupous mouse-derived MAbs (Fig. 2a), and the diffuse pattern was characteristic of the malaria-

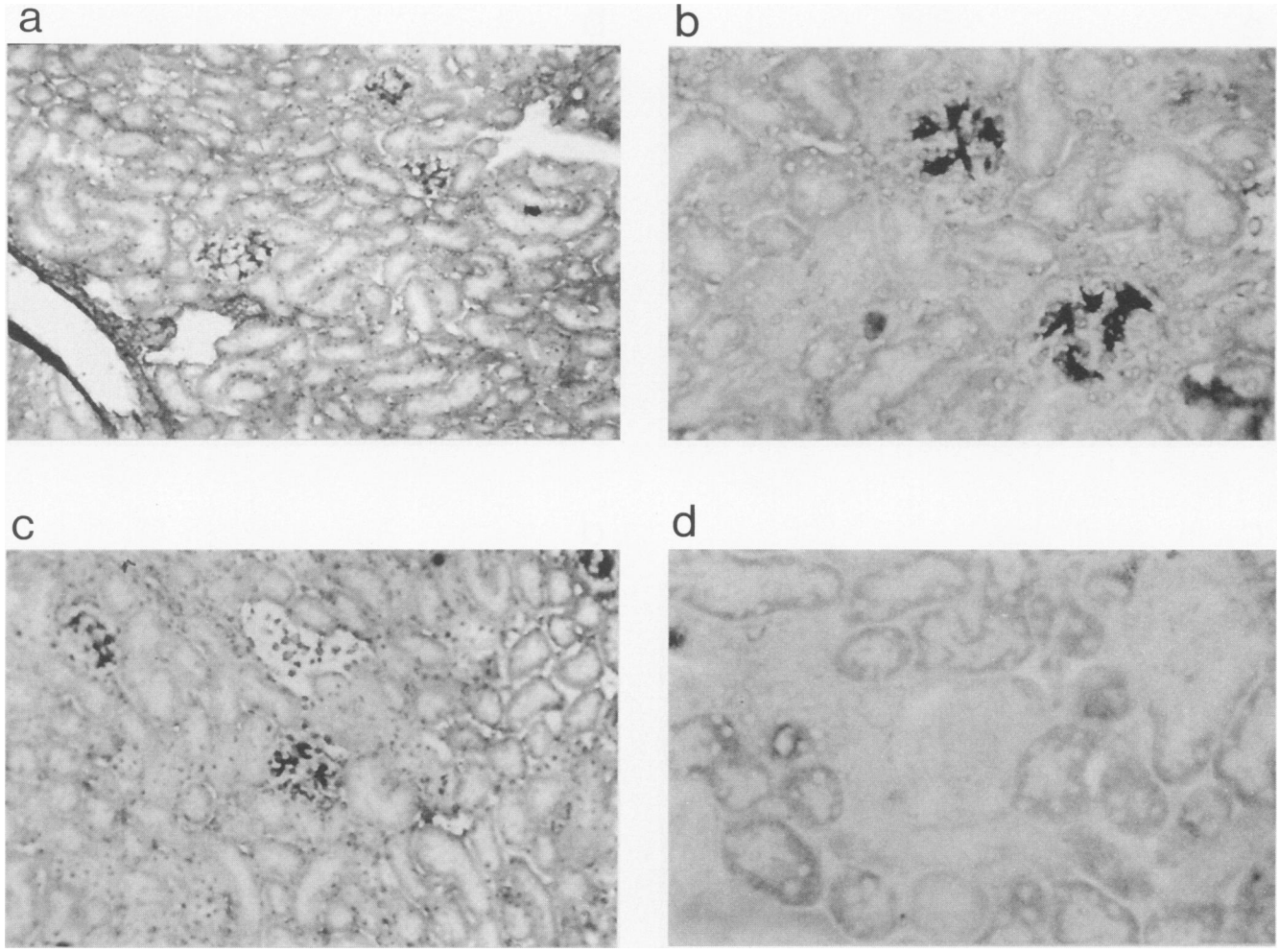

FIG. 1. Immunohistochemical (peroxidase) staining to show ex vivo attachment of DNA-binding MAbs to kidney sections prepared from *P. berghei*-infected mice. (a) Binding to mesangial areas of MAb IV-228, derived from an MRL/lpr mouse. (b) Similar mesangial binding of MA-1, obtained from a malarial mouse. (c) Staining of parasite pigment by MA-1. (d) No staining by MA-1 of kidney tissue from an uninfected mouse. No staining was seen in control preparations exposed initially to 1% normal mouse serum in PBS.

derived MAbs (Fig. 2b). However, two of the malaria-derived antibodies, MA-2 and MA-4, showed both the general diffuse staining pattern and the spotty staining pattern elicited by the MAbs from the lupous mice (Fig. 2c and d). There were no obvious differences in the staining of erythrocytes containing trophozoites or schizonts.

DISCUSSION

We have characterized a panel of DNA-binding MAbs from malaria-infected mice. Although DNA-binding antibodies in the sera of patients (1, 25, 34) and mice (31, 33) with malaria have been described, this is the first report describing MAbs derived from individuals with this infection.

All the DNA-binding MAbs produced were of the IgM isotype. This result reflects the pattern of serum DNA-binding antibodies: although all IgG subgroups are represented at the middle and later stages of infection, IgM antibodies persist throughout the infection (data not shown). In addition, we previously showed that malaria induces an increase in the levels of serum antibodies reactive with ssDNA rather than antibodies binding to dsDNA (31). In the present study, all of the malaria MAbs produced reacted preferentially with ssDNA and not with dsDNA. This result is contrary to the situation for SLE, in which dsDNA-binding antibodies are more common and are pathognomonic of the syndrome. None of the panel of malaria MAbs reacted with RNA, but they all reacted characteristically with polyribonucleotides, another property that distinguished them from the MAbs from the lupous mice. Complex cross-reactions of DNA-reactive antibodies are not unique to malaria but are found in MAbs derived from individuals with other infections. For example, IgM MAbs produced by hybridomas derived from leprosy patients bound to phenolic glycolipid I, a unique *Mycobacterium leprae* glycolipid, and to ssDNA, dsDNA, p(dT), and p(ADP-ribose) but not to RNA (19). In other studies, DNA-binding MAbs from BALB/c mice have also been found to be nonreactive with RNA (22).

As well as assessing the specificities of the malaria MAbs, we examined the expression of Ids that are commonly found on lupus-derived DNA-binding antibodies. Malaria antibodies were found to express some of the Ids found on lupus-derived antibodies, especially Id.V-88, which is a member of the Id.16/6 family (21, 28). The sharing of such Ids may indicate restrictions in the repertoire of variable-region Ig genes, implying

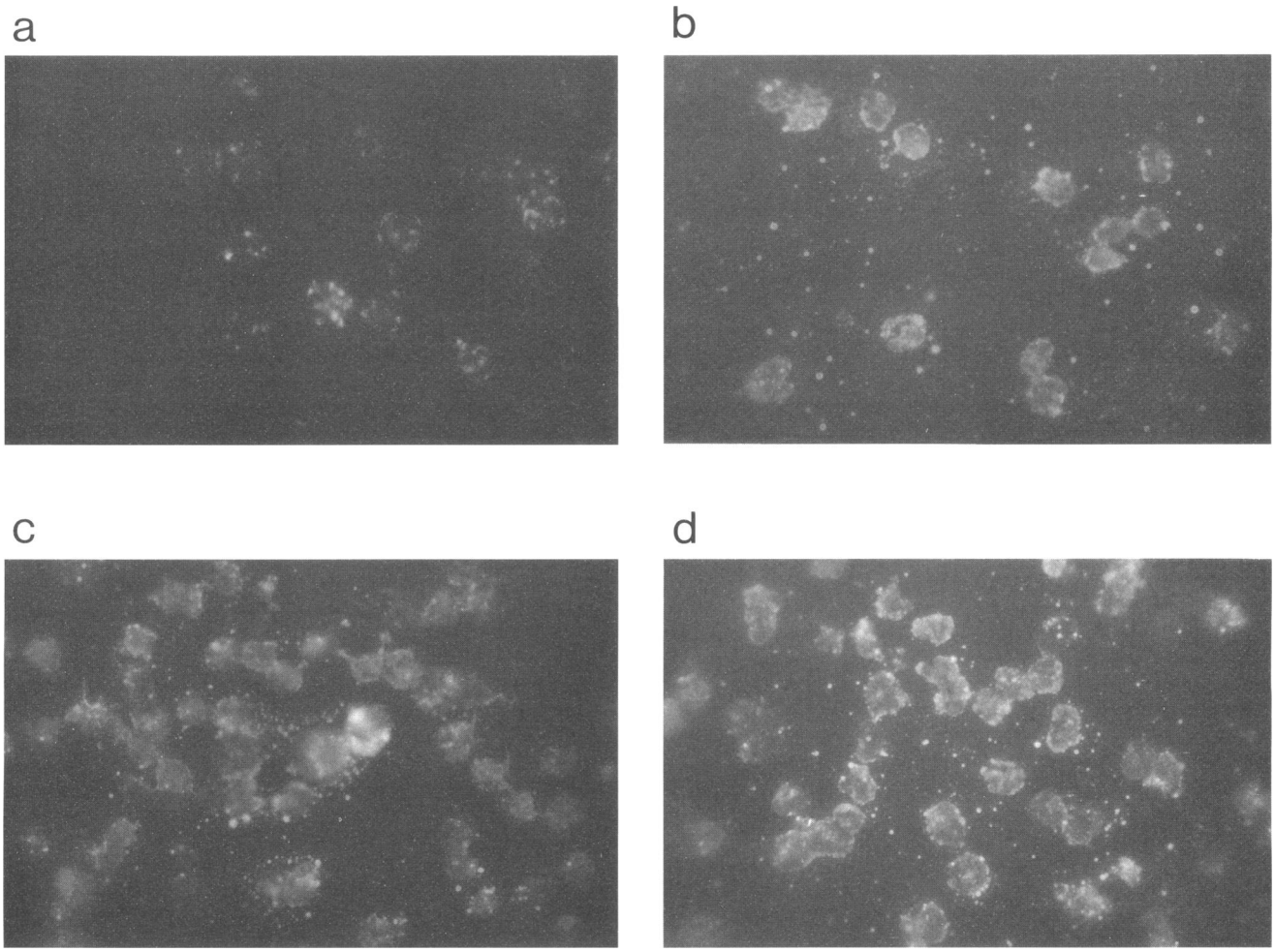

FIG. 2. Immunofluorescence staining to show the binding of DNA-reactive antibodies to parasitized erythrocytes separated from whole blood. (a) MAb IV-228 showed a distinct spotty staining pattern. (b) The typical malaria MAb MA-6 showed a more diffuse staining pattern. (c and d) Two of the malaria MAbs (MA-2 and MA-4) showed both of these patterns simultaneously. Uninfected cells showed only very faint diffuse staining with MAbs, a pattern which was quantitatively and qualitatively unlike the aforementioned patterns.

that the major cross-reactive Id families are the products of related or identical germ line genes rather than sets derived by somatic mutation. This implication is especially relevant for this panel of autoantibodies, since they are all IgM, and such molecules are less likely to undergo somatic changes than IgG or IgA antibodies (9). DNA-reactive antibodies that express Id.16/6 also occur in other parasitic infections, such as filariasis and schistosomiasis, in which the expression of this Id was found to increase as the infection progressed (30).

We derived the DNA-binding MAbs from mice with chronic malaria, but we failed in several attempts to isolate such antibodies from animals with an acute infection with *P. vinckei*. This result reflects the trends seen with DNA-reactive antibodies in the sera of infected mice, in which a chronic infection elicited titers of ssDNA-reactive antibodies (31) higher than those seen in an acute infection. DNA-binding MAbs derived from malarial mice reacted with kidney sections from the middle and late stages of infection but not those from uninfected mice or those from the early stage of infection. These results are comparable to the staining of similar kidney sections by the lupus-derived DNA-binding MAbs (31). There is evidence that dsDNA-binding antibodies are responsible for the development of nephritis during SLE. We discussed previously the possible involvement of DNA-binding antibodies in malarial nephritis (32); here we showed that ssDNA-binding antibodies may also bind to kidney tissues during a malaria infection and therefore may also be involved in the development of nephritis in this disease.

DNA-binding antibodies can bind to renal tissue in a variety of ways. Firstly, they may bind directly to the tissue itself or become lodged in the kidneys as preformed immune complexes (14). Alternatively, the antibodies may cross-react with highly anionic constituents of renal mesangial tissue or basement membrane, such as the glycosaminoglycans (7). It has also been found that these antibodies use other molecules, such as DNA (8) or DNA bound to histones (3) or fibronectin (17), as bridges to bind to tissue. Finally, a receptor for DNA has been found to exist on endothelial cells (6) and may aid in the formation of endothelial cell immune deposits in renal tissue.

The identity of the antigen which initiates the production of DNA-binding antibodies in both malaria and SLE remains unclear. During malaria, it is conceivable that these antibodies are directed towards a parasite-derived antigen which includes

DNA or which shares some epitopes with DNA. Why the MAbs react with phagocytosed parasite pigment is not known. It seems unlikely to us that DNA would persist as part of the crystalline pigment deposits in phagolysosomes; on the other hand, unequivocal cross-reactions of DNA-binding antibodies with parasite antigens have not, to our knowledge, been recorded. We have shown here that both lupus- and malaria-derived MAbs react with isolated parasitized erythrocytes. However, this reaction may represent binding to parasite DNA or to fragmented DNA within reticulocytes; this result is consistent with the fact that *P. berghei* (RC) prefers to invade reticulocytes rather than erythrocytes. It may be possible to further characterize these antigens by Western blotting (immunoblotting) and ultimately by sequence analysis. In this context, it is relevant that cross-reactions with intracellular proteins detected by immunoblotting are not uncommon for DNA-binding antibodies (4).

In conclusion, we identified and described a panel of MAbs which were derived from malaria-infected mice and which shared some properties with analogous DNA-reactive MAbs derived from lupous mice but which were clearly distinct from these. Their ability to bind to existing immune deposits in kidneys implicates them in the development of the nephritis that accompanies this infection. Although the antigen responsible for initiating the antibodies was not elucidated, it was found that DNA-binding antibodies from both the malaria and the lupus populations bound to parasitized erythrocytes, albeit with different staining patterns. The parallels between the pathobiological activities of the DNA-reactive antibodies in malaria and SLE are quite compelling, but there are still unanswered questions concerning the etiological relationship between the two diseases. Infection with malaria is known to compromise the development of idiopathic lupus disease in mice (10), and Butcher and Clarke (5) have hypothesized that genetic selection for resistance to malaria has led to an unavoidable susceptibility to autoimmune diseases, such as SLE, in populations that have migrated from areas in which malaria is endemic. It might be instructive to examine, for example, the influence of HLA-Bw53, shown by Hill and colleagues (11) to confer resistance to malaria, on susceptibility to SLE.

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