Table 2 Percentage of FITC-PNL binding cells in the axillary lymph nodes of mice stimulated with oxazolone

	Days after oxazolone	No. of mice	Mean
Expt 1	0	3	4.0±0.6
	3	4	3.4±1.5
	7	2	12.3±1.0
	13	1	13.9
	17	1	7.4
Expt 2	0	4	2.8±1.2
	4	4	3.3±0.9
	11	4	15.3±3.4
	15	4	19.2±6.0

PNL and FITC-PNL were prepared as described in the legend to Table 1. Mice were painted on the shaved flanks with 3 mg of 4-ethoxymethylene-2-phenyl-oxazolone (oxazolone, BDH) dissolved in 100 μl of ethanol. At various times after immunisation, mice were killed and cell suspensions from the draining axillary lymph nodes were prepared, they were washed twice and resuspended at a concentration of 10^7 per ml in PBS containing 0.05% sodium azide. The number of FITC-PNL binding cells was estimated as described in Table 1. Results are the mean ± s.d. from individual mice.

Table 3 Percentage of FITC-PNL binding cells in spleen cultured with LPS or PHA

	Time (h)		
	24	48	72
LPS	4.9±1.6	4.2±1.3	2.8±1.0
PHA	4.9±0.8	5.3±3.4	4.8±3.0

Spleen cells were cultured in RPMI1640 containing 10% fetal calf serum, 300 mM glutamine, 200 unit ml^{-1} penicillin and 150 μg ml^{-1} streptomycin, in 1 ml cultures at a concentration of 2×10^6 cells per ml with phytohaemagglutinin (PHA, Wellcome, 2.5 μg ml^{-1}) or 4×10^6 cells per ml with lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Difco, 50 μg ml^{-1}). After culturing (at 37 °C in an atmosphere of 7% CO_2, 10% O_2, 83% N_2) for 24, 48 or 72 h, cells were collected and the numbers of FITC-PNL binding cells were estimated as described in Table 1. Results are the mean ± s.d. from four replicate cultures. Control cell suspensions of uncultured spleen revealed 6.0±2.0, 5.5±1.7 and 4.7±1.1% FITC-PNL binding cells at 24, 48 and 72 h respectively. A control thymocyte suspension done at 72 h revealed 82% PNL binding cells.

The origin of cells in germinal centres has not been fully determined. Weissman[12] has shown that there are some T cells present by the use of fluorescinated anti-T cell antiserum. However, neither the numbers nor the pattern of dispersion of these cells corresponds to that seen with PNL. Thus the PNL-positive cells are unlikely to be mainly T lymphocytes. In the same study Weissman demonstrated that a substantial proportion of the area of germinal centres was immunoglobulin positive. This finding must be viewed with some caution before it is assumed that many or all of the cells in germinal centres are surface immunoglobulin-positive B lymphocytes. It is known that, in at least some germinal centres, there are dendritic reticular cells which bind antigen–antibody complexes and which have cell membranes with vast processes on which the complexes are to be found[13]. Preliminary studies in this laboratory with double staining techniques on cell suspensions from Peyer's patches have shown that some of the PNL-positive cells appear to be surface immunoglolobulin positive, whereas others are not obviously so. Thus the anatomic origins of PNL-positive germinal centre cells are not clear, although it seems likely that some or all of them are B lymphocytes.

It is an intriguing coincidence that cortical thymocytes and germinal centre cells bind PNL. These observations, together with Reisner's suggestion[4] that PNL binding is a marker of immaturity among T cells and cells of the haematopoietic stem cell population, again raise the possibility that germinal centres harbour a population of immature B cells[14,15]. Use of PNL to separate germinal centre cells should allow their properties and functions to be characterised further.

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Protective monoclonal antibodies recognising stage-specific merozoite antigens of a rodent malaria parasite

Robert R. Freeman, Avril J. Trejdosiewicz & George A. M. Cross

Department of Immunochemistry, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, UK

Immunity to malaria is mediated, at least in part, by antibody. Resistance to infection has been passively transferred with immune serum or its immunoglobulin fraction in human[1], simian[2] and rodent[3,4] malaria. However, because of the structural and antigenic complexity of the malaria parasites[5,6], it has proved difficult to identify and characterise those parasite antigens against which protective antibody is directed. We have produced several hybrid cell lines secreting monoclonal antibodies against the rodent malaria parasite, *Plasmodium yoelii*, and we now report that, of the antibodies tested, only those specific for antigens exclusive to the merozoite were protective in passive transfer experiments. Other anti-*P. yoelii* monoclonal antibodies, apparently recognising antigens in the membrane of the infected erythrocyte, were not protective on passive transfer. The protective monoclonal antibodies should be useful in the isolation of the important antigens of this parasite.

Spleen cells from two *P. yoelii*-immune BALB/c mice were fused with P3-NS1/1-Ag4-1 myeloma cells in the presence of polyethylene glycol[7]. The cells were dispensed into 144 tissue culture wells in 2-ml volumes of hypoxanthine-aminopterine-thymidine-selective medium[8], using RPMI-1640 medium supplemented with 10% fetal calf serum as a base[9]. After 10 days, the culture supernatants were tested for *P. yoelii*-specific antibody by indirect immunofluorescence (IIF). Of 143 cultures tested, 38 were positive, 20 producing antibody apparently directed against the infected erythrocyte membrane and 18 producing antibody against the parasite itself. From the initial screening, five distinct IIF specificities were detected. Cultures representative of each of the IIF specificities were expanded in selective medium without aminopterine, and hybridoma lines were subsequently cloned in semi-solid agar overlayed with medium.

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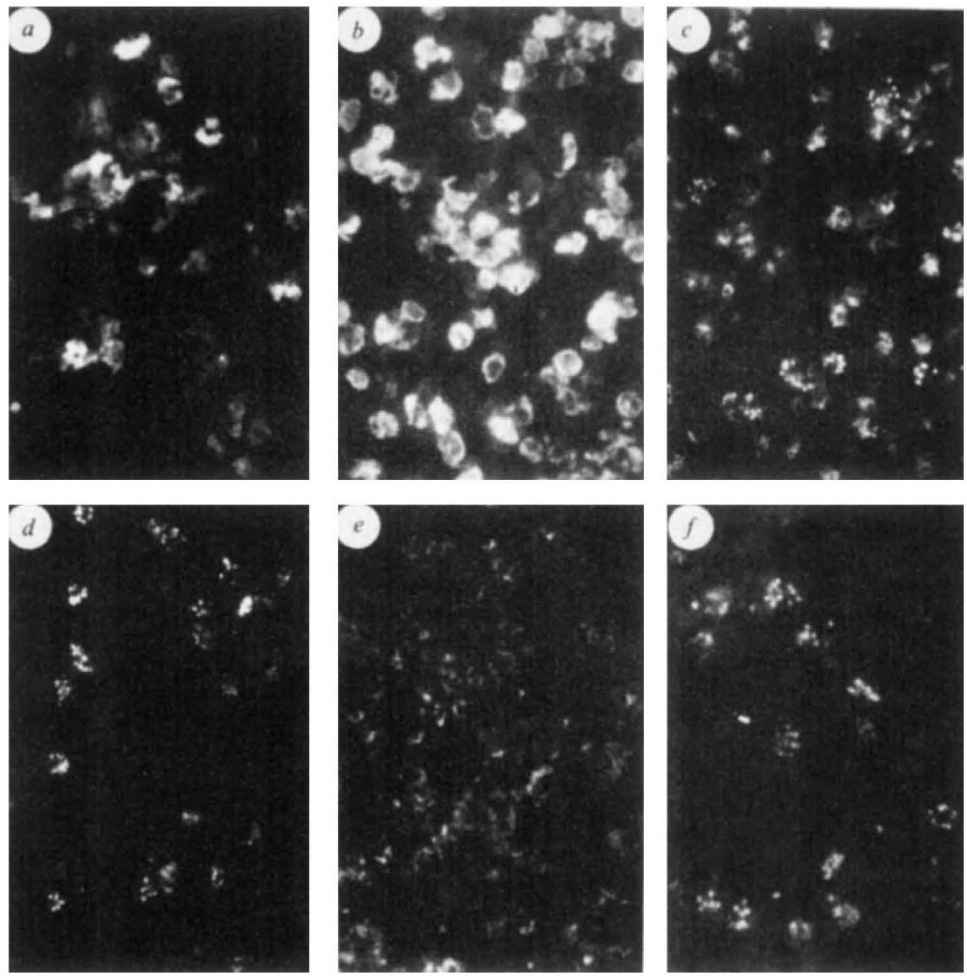

Fig. 1 IIF staining reactions produced by: *a*, whole anti-*P. yoelii* immune serum diluted 1 in 1,000, and *b–f*, 1 in 10,000 dilutions of sera from mice carrying ascites tumours derived from cloned hybridoma cell lines: *b*, WIC 25.1; *c*, WIC 25.23; *d*, WIC 25.37; *e*, WIC 25.54; *f*, WIC 25.77. Supernatants from cultures of these lines produced IIF reactions identical to those produced by the sera. To produce monoclonal antibodies, 2×10^8 spleen cells from BALB/c mice recovered from mild *P. yoelii* 17X infection and rechallenged three times with virulent *P. yoelii* (YM strain) were mixed for fusion with 2×10^7 P3-NS1/1-Ag4-1 myeloma cells[9]. To prepare antigen for the IIF test, BALB/c mice were infected with virulent *P. yoelii* 24 h before subcutaneous injection with cyclophosphamide (200 mg per kg). After 4 days, when parasitaemia was >60%, infected blood was enriched for schizonts by centrifugation through isopaque/Ficoll[12] (2,000*g* for 20 min at 20 °C). The enriched upper layer of cells was washed in phosphate-buffered saline (PBS), diluted 1 in 100 in PBS, and applied dropwise to glass slides. The antigen preparation was allowed to dry at room temperature, then slides were stored at −20 °C. Giemsa staining of preparations showed that all red cells were parasitised (30% schizonts, 70% trophozoites approximately). Free merozoites were also observed. The procedure for the IIF test has been described elsewhere[13]: in the present study, background fluorescence was minimised by using immunoabsorbant-purified rabbit anti-mouse Ig. (×180).

The five cloned lines secreted antibodies covering the range of IIF specificities originally detected. Whereas polyspecific serum from immune mice produced a generalised staining of *P. yoelii*-infected erythrocytes in the IIF assay, the monoclonal antibody specificities produced characteristic, restricted patterns of fluorescence (Fig. 1). Thus, with specificity 25.1, the membranes of a subpopulation of infected erythrocytes fluoresced, whereas specificity 25.54 bound to the membranes of all infected erythrocytes with a distinct patchy staining pattern. Specificity 25.23 bound to an antigen common to all developmental stages of the blood form of the parasite. Specificities 25.37 and 25.77 bound to antigens only present in merozoites, free and within mature schizonts. All merozoites in the antigen preparation apparently reacted with the two merozoite-specific antibodies, but the possibility that a minor subpopulation of merozoites failed to react could not be excluded.

Table 1 Characteristics of the anti-*P. yoelii* monoclonal antibodies secreted by five cloned hybridoma lines

Hybridoma line	IIF specificity of secreted antibody	IIF cross-reactivity against *P.v.petteri*	Immunoglobulin subclass
WIC 25.1	Infected erythrocytes	+	IgG2a
WIC 25.23	Trophozoites, schizonts and merozoites	+	IgG2a
WIC 25.37	Merozoites	−	IgG1
WIC 25.54	Infected erythrocytes	+	IgG3
WIC 25.77	Merozoites	+	IgG2a

IIF specificity was determined by testing against *P. yoelii*, YM strain. For immunoglobulin subclass determination, concentrated culture supernatant samples were tested by double diffusion in agar against rabbit antisera specific for the mouse immunoglobulin subclasses IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA.

The antibody products of the five lines were characterised with respect to immunoglobulin subclass by double diffusion using subclass-specific antisera. All were found to be of the IgG class (Table 1). They were also screened for cross-reaction against uninfected BALB/c erythrocytes and against a heterologous rodent malaria parasite, *Plasmodium vinckei petteri*, by IIF. None reacted with the uninfected erythrocytes but all except the merozoite-specific antibody secreted by line WIC 25.37 cross-reacted with *P.v.petteri* antigens (Table 1).

Serum pools containing high titres of anti-*P. yoelii* monoclonal antibodies were obtained from hybridoma tumour-bearing BALB/c mice (Table 2). These sera were tested in passive transfer experiments for their effect on the course of virulent *P. yoelii* infection (Fig. 2). Mice receiving monoclonal antibody specificities 25.1 and 25.54, apparently directed against infected erythrocytes, were not protected against *P. yoelii*. Neither was protection transferred with serum containing a high titre of specificity 25.23, directed against an antigen common to all developmental stages of the asexual erythrocytic parasite (trophozoites, schizonts and merozoites). However, transfer of sera containing the antibodies specific for late-appearing merozoite antigens, specificities 25.37 and 25.77, resulted in inhibition of *P. yoelii* parasitaemia, and none of these mice died. Whole immune serum was even more effective, causing the elimination of patent parasitaemia within 48 h of transfer. The protective activity of the monoclonal antibodies was not restricted to one IgG subclass, as specificity 25.37 was an IgG1 and specificity 25.77 was an IgG2a.

The eventual recovery of those groups treated with monoclonal anti-merozoite antibody was probably not mediated by the transferred serum, but rather by the recipients' immune

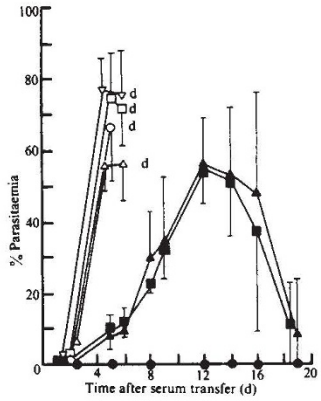

Fig. 2 The course of *P. yoelii* infection in groups of five BALB/c mice injected intraperitoneally with 0.5 ml of normal mouse serum (○), *P. yoelii*-hyperimmune serum (●), or serum from mice carrying tumours derived from cloned hybridoma lines: WIC 25.1 (△), WIC 25.23 (□), WIC 25.37 (▲), WIC 25.54 (▽) and WIC 25.77 (■). Serum was transferred when parasitaemia had reached 0.01–0.1%. Mean survival times are indicated (d). The vertical bars indicate standard deviations.

response. The direct effect of the transferred antibody was seen during the week following transfer, when the normally fulminating *P. yoelii* YM infection was retarded, following a course more typical of self-limiting *P. yoelii* 17X infection.

Inhibition of red cell invasion by merozoites may involve the blocking by antibody of receptor determinants on the merozoite surface. A polyspecific antiserum would be expected to be more efficient in receptor blocking than a monospecific antiserum. This may explain the greater degree of protection transferred with whole hyperimmune serum than with serum containing a single anti-merozoite specificity. An interesting alternative possibility is that the delayed parasitaemia observed in mice treated with specificities 25.37 and 25.77 was due to the emergence of a population of merozoites not reacting with these antibodies.

It is not known whether monoclonal specificities 25.37 and 25.77 recognise distinct merozoite antigens or different determinants on the same antigen. They give similar IIF staining reactions against *P. yoelii* (Fig. 1), but the observation that specificity 25.77 is cross-reactive with *P.v.petteri* indicates that this antibody possesses a binding specificity distinct from that of the other protective monoclonal antibody species. Using monoclonal antibodies and immunoprecipitation, we are now in the process of identifying the parasite antigens involved.

Our findings do not support the view that antibodies specific or parasite antigens present in the membrane of the infected erythrocyte are protective[10], although they do indicate the presence of at least one erythrocyte membrane-localised *P. yoelii* antigen, against which a substantial but apparently non-protective antibody response is made in the immune host. However, we have tested only two membrane-specific monoclonal antibodies, and our results do not rule out the possibility that other membrane-specific antibodies may prove protective. Furthermore, although specificities 25.1 and 25.54 bound to the membranes of infected erythrocytes in fixed smears, they did not bind to infected erythrocytes in suspension, and so the antigens recognised by these antibodies are probably not externally expressed on infected erythrocytes. Nevertheless, protection was transferred with the anti-merozoite monoclonal antibodies, and it therefore seems that in the case of *P. yoelii*, as with *Plasmodium knowlesi*[11], antibody-mediated protection against malaria acts at the level of the free merozoite, inhibiting invasion of host erythrocytes.

Table 2 IIF titres of sera used in passive transfer experiments

Serum source	Anti-*P. yoelli* IIF titre
BALB/c mice carrying hybridoma WIC 25.1	1:30,000
BALB/c mice carrying hybridoma WIC 25.23	1:32,000
BALB/c mice carrying hybridoma WIC 25.37	1:128,000
BALB/c mice carrying hybridoma WIC 25.54	1:30,000
BALB/c mice carrying hybridoma WIC 25.77	1:128,000
Immune BALB/c mice*	1:70,000
Normal BALB/c mice	<1:20

Anti-*P. yoelii* IIF titre is expressed as the highest dilution giving positive fluorescence.

* Donors had recovered from mild *P. yoelii* 17X infection, and serum was taken 7 days after challenge with virulent *P. yoelii* (YM strain).

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Copper metallothionein, a copper-binding protein from *Neurospora crassa*

Konrad Lerch

Biochemisches Institut der Universität Zürich, Zürichbergstrasse 4, CH-8032 Zürich, Switzerland

Copper is an essential constituent of many proteins which participate in biologically important reactions[1]. In contrast to iron, where different metal storage and transport proteins have been extensively characterised, the existence of copper proteins serving such functions is still a matter of controversy[2–8]. Studies on the biosynthesis of tyrosinase from *Neurospora crassa* with respect to the copper status of this fungus have shown that this organism accumulates copper with the concomitant synthesis of a small molecular weight copper-binding protein. This protein is now shown to have a striking sequence homology to the zinc- and cadmium-containing metallothioneins from vertebrates[9]. Growth experiments suggest that this molecule fulfills several important physiological functions in this organism such as copper storage, copper detoxification and provision of copper for tyrosinase.

When *N. crassa* is grown in the presence of 0.5 mM of $CuSO_4$ in the culture medium most of the intracellular copper is found in a high molecular weight fraction during the logarithmic growth period (Fig. 1). However, in the stationary phase about 10% of the copper taken up by the cells accumulates in the form of a low molecular weight protein (Fig. 1). This copper-binding protein was isolated from lyophilised mycelium (obtained as described in Fig. 1 legend) by gel filtration of the crude extract on Sephadex G-50 and subsequent chromatography on DEAE-cellulose. The pure protein is composed of only seven different amino acids with a strikingly high content of cysteine (28%), serine (28%) and glycine (24%). The amino acid composition is: Asx, 2.9; Ser, 6.9; Gly, 6.0; Ala, 1.0; Cys, 6.9 (determined as carboxymethylcysteine); Lys, 1.0. The protein binds a total of 6 g atoms of copper per mw of 2,200. The copper-free apoprotein can be obtained by exposure of the holoprotein to low *p*H (<*p*H 1.0) and subsequent metal removal by gel filtration. The homogeneity of the molecule was further corroborated by the

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