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Monoclonal antibodies from Epstein-Barr virus-transformed lymphocytes of common marmosets (*Callithrix jacchus*) immune to malaria

D.A. Johnston [1,2], A.M. Knight [1], B.A. Naylor [2], N. Wedderburn [2] and G.H. Mitchell [1]

[1] *Department of Immunology (MS 2), United Medical and Dental Schools of Guy's and St. Thomas', The Medical School, Guy's Hospital, London Bridge, London. SE1 9RT, U.K., and* [2] *Department of Pathology, Hunterian Institute, Royal college of Surgeons of England, Lincoln's Inn Fields, London WC2A 3PN, U.K.*

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The B lymphocytes of the common marmoset *Callithrix jacchus* can be immortalised by infection with Epstein-Barr virus (EBV) in vitro (Desgranges et al., 1976). *C. jacchus* is susceptible to infection with the blood stages of several species of malaria parasite including the line designated MVF1 (Mitchell et al., 1988) from which it recovers and shows immunity to reinfection. By exploiting these two phenomena, EBV-transformed, marmoset lymphoblastoid cell lines secreting antibodies to malaria parasite antigens have been generated and cloned. We believe this to be the first time that monoclonal antibodies (MAbs) have been raised from common marmosets. Since numerous and diverse human pathogens can infect this small primate in the laboratory, these methods may prove generally applicable for the generation of MAbs whose specificities derive from immune responses to infection.

Key words: Common marmoset; Epstein-Barr virus; Feeder cell; Malaria; Monoclonal antibody

Correspondence to: G.H. Mitchell, Department of Immunology (MS 2), The Medical School, Guy's Hospital, London Bridge, London SE1 9RT, U.K.

Abbreviations: DMSO, dimethyl sulphoxide; EBV, Epstein-Barr virus; EDTA, ethylenediaminetetraacetic acid; EGTA, 1,2-di(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; FCS, heat-inactivated foetal calf serum; FITC, fluorescein *iso*-thiocyanate; Ig, immunoglobulin; LCL, lymphoblastoid cell line; MAb, monoclonal antibody; ND, not done; NP40, Nonidet P-40; PBL, peripheral blood lymphocyte; PI, post infection; PMSF, phenylmethylsulfonyl fluoride; PPO, 2,5-diphenyloxazole; PT, post transformation; RBC, red blood cell; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; T, transformation; TES, *N*-Tris(hydroxymethyl)methyl-2-amino-ethanesulphonic acid; TLCK, *N*α-tosyl-L-lysyl chloromethane hydrochloride; Tris, tris(hydroxymethyl)methylamine.

Introduction

Monoclonal antibody (MAb) technology has greatly facilitated the identification of antigens in complex pathogens. MAbs that are produced from animals which show protective immunity following infection may be of particular relevance to such analyses.

The ability of Epstein-Barr virus (EBV) to transform human B lymphocytes (Pope et al., 1968) has been exploited in the production of human monoclonal antibodies to antigens from diverse viral, bacterial and eukaryotic pathogens (see Crawford (1986) for review). Although B lymphocytes from the common marmoset, *Calli-*

thrix jacchus (a small, neotropical primate) may similarly be immortalised by infection with EBV in vitro (Desgranges et al., 1976), exploitation of this phenomenon for MAb production has not previously been reported.

We have been evaluating *C. jacchus* as a laboratory host for *Plasmodium vivax*, a widely distributed and clinically important human malaria parasite. In one marmoset which had been infected with a laboratory line of *P. vivax*, we first observed and then isolated a morphologically altered, virulent malaria which was initially considered to be a marmoset-adapted, mutant of *P. vivax* (Mitchell et al., 1988). Recent gene sequence data refute this assumption and suggest that a second species of malaria parasite contaminated the original inoculum; formal reidentification is in hand (Waters et al., in preparation). The immunobiology of this malaria infection, designated MVF1 (Marmoset Virulent Form 1), has been extensively studied in *C. jacchus*. Primary infections with MVF1 are self-limiting and generate high titres of anti-malarial immunoglobulin G (IgG). Challenge of self-cured animals results in a low grade, abbreviated parasitaemia accompanied by a further rise in antibody titres (Mitchell et al., 1988). We have used EBV to immortalise splenic lymphocytes from common marmosets which have recovered from infection with MVF1 malaria and have cloned the resulting lymphoblastoid cell lines (LCLs). Using X-irradiated, EBV-transformed LCLs raised from naive marmosets as feeder cells, monoclonal, marmoset LCLs were generated which were shown to secrete antibodies directed against MVF1 malaria antigens. These MAbs, whose specificities are derived from a protective immune response, will further our understanding of the immunobiology of this model system.

Materials and methods

Cell culture

Marmoset LCLs were routinely maintained in RPMI 1640 medium (Gibco/Flow) supplemented with penicillin (Gibco), streptomycin (Gibco) and heat-inactivated foetal calf serum (FCS) (Seralab) at 100 IU/ml, 100 μg/ml and 10% respectively, hereinafter referred to as culture medium. For transformation, fungizone (Gibco) and nystatin (Gibco) were added to culture medium at 5 μg/ml and 20 U/ml respectively and penicillin, streptomycin and FCS levels were doubled. Culture medium supplemented with 20% FCS was also used to establish clonal cultures. Cultures were maintained at 37°C in a water-saturated atmosphere of 5% CO_2 in air.

Transforming virus

EBV was obtained from the supernatants of the EBV-transformed *Saguinus oedipus* LCL B-95-8 (Miller et al., 1972) (kind gift of Dr. D. Crawford) and the EBV-transformed *Callithrix jacchus* LCL M81 (Desgranges et al., 1976) (kind gift of Dr. C. Desgranges).

The B-95-8 and M81 LCLs were routinely maintained in tissue culture flasks (Nunc) and expanded, twice weekly, by two-fold dilution of the cell suspension. Prior to harvesting for virus, cultures were left, without feeding, for 10 days.

As *Callithrix* B lymphocytes are less susceptible to transformation by EBV than are human (C. Desgranges, personal communication, see also Henderson et al. (1977)), virus-containing supernatants were concentrated 100-fold by ultracentrifugation prior to use. Thus supernatants were first clarified by low-speed centrifugation followed by filtration (0.45 μm) and then spun at 60,000 × *g* for 1 h at 4°C to pellet EBV. The virus-rich pellet was resuspended in fresh culture medium (M81) or clarified supernatant (B-95-8, C. Desgranges, personal communication) to 1% of the starting volume and stored in liquid N_2.

Marmosets

Common marmosets (*Callithrix jacchus*) were bred in a closed colony to which there have been no additions since 1970. None of the animals used in these experiments had previously been exposed to malaria parasites or to EBV infection.

Parasite infection in lymphocyte donors

The marmoset-adapted malaria parasite line MVF1 was used throughout. Parasites were handled and enumerated as previously described (Mitchell et al., 1988).

Lymphocytes for transformation were obtained from the spleens of two animals. Malaria was

induced in both by inoculation of infected blood and both showed typical courses of primary infection (Mitchell et al., 1988).

Marmoset D553 (16 months at time of infection) had a single infection and showed a peak parasitaemia of 1.6% at day 27 post infection (PI) which had fallen to barely detectable levels at the time the animal was killed (day 350 PI).

Marmoset 220 (11 months at time of infection) had a similar initial infection but was rechallenged six times between days 262 and 339 PI and killed 31 days after the final rechallenge.

Only the first of these rechallenges produced a detectable parasitaemia.

Transformation of splenic lymphocytes

After killing spleens from marmosets D553 and 220 were removed to serum-free culture medium, or to Hanks' balanced salt solution, for transport, rinsed in serum-free culture medium, minced and pushed through a stainless steel mesh (aperture 600 μm). Lymphocytes were purified on Ficoll-Paque (Pharmacia), washed three times in serum-free culture medium, resuspended at 10^7 cells/ml in 100 × concentrated EBV suspension (B-95-8 or M81) and incubated at 37 °C for 1 h. Cell suspensions were then diluted to 10^6/ml by the addition of fresh culture medium supplemented with fungizone, nystatin and extra penicillin, streptomycin and FCS (as above) and seeded into 24 well plates (Linbro).

Surplus isolated, washed lymphocytes were cryopreserved and stored in liquid N_2 using standard protocols. These were later resuscitated and, following a 6 h recovery period in culture medium supplemented with 20% FCS, transformed as described above.

Primary cultures

After transformation, cultures were left undisturbed for 9–15 days and then, at 3–10 day intervals (depending on the rate of medium acidification) 50% of the culture medium was exchanged for fresh. This regime was continued until definite population expansion was apparent. Depending on their growth rate, cultures were then expanded at 4–19 day intervals (mean 7 days) by 2–3-fold dilution. When lines demonstrated consistent growth, a regime of expansion by two-fold dilution every 3–4 days was adopted.

Primary cultures were screened for anti-malaria antibody at 13 days post transformation and at 2-weekly intervals thereafter.

Establishment of feeder cell lines

X-irradiated, EBV-transformed LCLs from a normal animal were used as feeder cells. To generate these lines, peripheral blood lymphocytes (PBLs) from a normal marmoset (designated 210) were isolated on Ficoll-Paque, washed and transformed as described above. Once consistent growth was apparent, feeder cell lines were maintained by twice-weekly dilution to 5×10^5 cells/ml. Both B-95-8- and M81-derived stocks of EBV were used to generate feeder cell lines (designated 210B and 210M respectively) so that antibody-secreting cells could be cloned in the presence of feeder cells carrying the same strain of virus.

Cloning protocol and maintenance of resulting lines

The appropriate feeder cell line was X-irradiated at 2000–4000 R and then diluted with sufficient fresh culture medium and FCS to yield 5×10^5 cells/ml in medium supplemented with 20% FCS (thus the medium was partly pre-conditioned by feeder cells). The cells to be cloned were then added in a minimal volume of culture medium and 200 μl aliquots of the resulting cell suspension were seeded into flat-bottomed, 96 well plates (Nunc). At 2-week intervals, 50% of the medium in the wells was exchanged for fresh culture medium. In some cases, an additional 10^5 X-irradiated feeder cells were added to each well at the first medium change. Antibody-producing LCLs were routinely established from primary cultures seeded at the equivalent of one or ten cells/well.

Anti-malaria antibody-secreting LCLs were transferred to 1 ml culture medium in a well of a 24 well plate (Linbro) and expanded, by a two-fold dilution of the cell suspension, once or twice weekly. Once LCLs had been expanded to 4 ml or beyond, they were recloned. Established clones were maintained by two-fold dilution every 3–4 days.

Screening protocol

Culture supernatants were screened for anti-malaria antibody by indirect immunofluorescence on dried, unfixed thick films of washed, MVF1-infected marmoset red blood cells (RBC) as previously described (Mitchell et al., 1988). To conserve antigen and to minimise screening times, a 'cocktail' of FITC-conjugated rabbit anti-human IgG (Dako) and FITC-conjugated sheep anti-human IgM (Serotec) (which had previously been screened for reactivity with marmoset IgG and IgM respectively) was used to detect bound marmoset antibody.

Characterisation of monoclonal antibodies:

Marmoset anti-MVF1 monoclonal antibodies (and immune marmoset sera) were characterised by immunoprecipitation of antigens from a crude lysate of radiolabelled, MVF1-infected RBCs. Precipitated antigens were separated by SDS-PAGE and identified by fluorography.

1 ml of blood from an MVF1-infected marmoset (parasitaemia approximately 1%) was washed in serum-free malaria culture medium (RPMI 1640 supplemented with 0.4 M TES, 1 mg/ml D-glucose, 10 μg/ml hypoxanthine and 2 IU/ml heparin (Leo Laboratories)) and then incubated in 10 ml of serum-free, methionine-free malaria culture medium (Gibco 'Selectamine' plus additives as above) supplemented with 0.2–1 mCi [^{35}S]methionine (Amersham) under an atmosphere of 90% N_2, 5% O_2, 5% CO_2 for 1–3.5 h. RBCs were then extensively washed and stored at $-70\,^{\circ}$C. Cell lysates were prepared and immunoprecipitations performed by the method of Holder and Freeman (1982) using 5×10^5 cpm of lysate and 750 μl of culture supernatant or 5 μl of immune marmoset serum per reaction. Immunoprecipitated complexes were solubilised and then separated by SDS-PAGE (reducing conditions) using standard protocols. Gels were fixed, stained with Coomassie blue, destained, impregnated with 22.2% PPO in DMSO and dried using standard protocols. Dried gels were placed against pre-flashed X-ray plates (Kodak X-Omat) and incubated at $-70\,^{\circ}$C for 1–4 weeks. To facilitate photographic documentation, bands on developed fluorographs were intensified by chromium enhancement using standard photographic techniques.

Results

Transformation and primary cultures

Fresh splenic lymphocytes from marmosets D553 and 220, and frozen, resuscitated splenic lymphocytes from marmoset 220, were sucessfully transformed, and monoclonal, anti-malaria antibody-secreting LCLs generated from each (designated transformations (T) 3, 4 and 5 respectively). PBLs from normal marmoset 210 were also successfully transformed to provide feeder cell lines for cloning.

For the first 6–8 weeks after transformation, cultures showed considerable metabolic activity, necessitating regular medium changes, but little or no increase in cell numbers. Cultures were first expanded at 10.5, 10, 9 and 5 weeks post transformation (PT) (T3, T4, T5 and 210B/210M respectively) and routine, twice weekly expansion was initiated at approximately 15 and 8.5 weeks PT (T3-5 and 210B/210M respectively). Subsequent experience from further transformations (data not shown) suggests that expansion of T3-5 could have commenced earlier. FCS levels were reduced to 10% at 6–13 weeks PT for T3-5, and to 15% at 8 weeks and to 10% at 10 weeks PT for 210B/210M.

Anti-malaria antibody was observed in the supernatants from primary cultures when first tested at 13 days PT and was still present at 133, 110 and 93 days PT in T3, T4 and T5 respectively. Supernatants from primary cultures from T4 and T5 were not tested beyond this date; those from T3 lost detectable anti-malaria activity sometime between 133 and 140 days PT.

Cloning

X-irradiated, EBV-transformed PBLs from normal marmosets proved to be effective feeder cells, permitting the clonal growth of EBV-transformed, anti-malaria antibody-secreting splenocytes. Irradiated marmoset lymphoblastoid cells showed little tendency to break up, remaining morphologically intact for many weeks after irradiation. Thus, growing clones could not reliably be detected by microscopic observation. All wells on all plates were, therefore, screened by immunofluorescence.

Anti-malaria antibody secreting LCLs were sucessfully raised from primary cultures subcul-

TABLE I

SUMMARY OF DATA FROM CLONING PRIMARY CULTURES OF EBV-TRANSFORMED MARMOSET SPLENOCYTES

Culture	Seeded (days post transformation)	Seeding density (cells/well)	Screened for anti-malaria antibody (days post cloning)	Number of wells positive for anti-malaria antibody [a]	Number of anti-malaria antibody-containing wells successfully expanded [a]
T3-1	16	1	53	0/45	0
	16	10	74	3/240	0
	16	100	39, 74	15/120	1
T3-2	23	1	92, 105	5/300, 4/360	3, 2
	23	10	76	11/180, 5/180	10, 4
T3-3	66	1	70	4/120, 0/120	0, 0
	66	10	70 98	7/60, 2/60	2, 1
T4-1	22	1	70	0/120, 6/120	0, 1
	22	10	70	0/60, 3/60	0, 0
T5-1	57	1	71, 85	12/240, 3/240	3, 1
	57	10	71, 85	12/60, 6/60	6, 0

[a] Results from subcultures grown in parallel but derived from separate primary cultures are shown separated by commas.

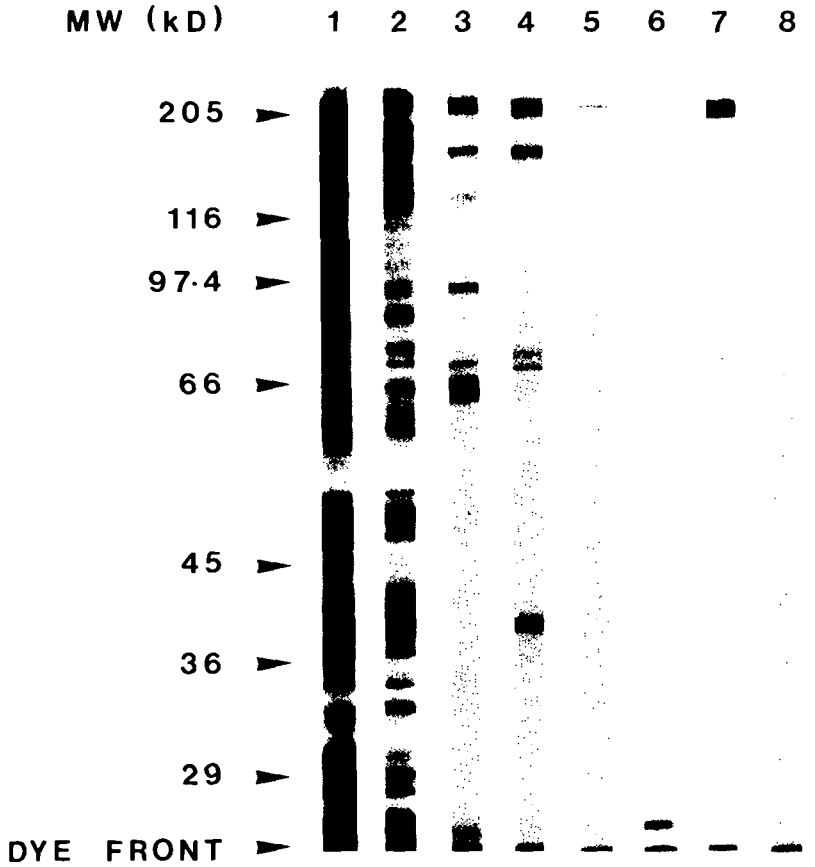

Fig. 1. Fluorographs illustrating the specificities of antibodies secreted by EBV-transformed, marmoset LCLs as determined by immunoprecipitation from a crude lysate of ^{35}S methionine-metabolically-labelled, MVF1-infected marmoset red blood cells using: track 1: immune marmoset serum; tracks 2–3: supernatants from uncloned primary cultures; track 4: supernatant from a 100 cells/well subculture; tracks 5–7: supernatants from monoclonal LCLs; track 8: control (RPMI 1640 + 10% FCS).

TABLE II

SUMMARY OF DATA FROM RECLONING EBV-TRANSFORMED, MARMOSET, ANTI-MALARIA ANTIBODY SECRETING LCLs

Culture	Seeding density (cells/well)	Screened for anti-malaria antibody (days post cloning)	Number of wells positive for anti-malaria antibody [a]
T3-5	2	61	21/240
T3-6	1	60, 74, 92	0/180, 0/240 0/240, 1/240
T3-7	1	132	10/120, 8/60
T3-8	1	60	45/180, 28/120
T3-10	0.3	43	9/180, ND [b]
	1	62	29/240, 1/60
	3	62	ND, 0/60
T5-2	1	60	1/180
T5-3	0.3	56	ND, 0/180
	1	56	0/180, 0/240
	3	56	0/60, 0/60

[a] Results from subcultures grown in parallel but derived from separate parent cultures are shown separated by commas.
[b] Not done.

tured at 1 or 10 cells/well at between 22 and 66 days PT (latest time attempted). Of subcultures initiated at 16 days PT, only wells seeded with 100 cells gave rise to LCLs. FCS levels were reduced to 10%, and supernatants screened for anti-malaria antibody, at 6–13 and 8–15 weeks post seeding respectively. Approximately 2% (range 0–5%) of wells on plates seeded from primary cultures at the equivalent of one cell/well were found to be positive for anti-malaria antibody. However, not all of these were successfully expanded to yield LCLs. Data from these cultures are summarised in Table I.

On recloning, FCS levels were reduced to 10%, and supernatants screened for anti-malaria antibody, at 4 and 6–19 weeks post seeding respectively. Cloning efficiency, as determined by Poisson distribution analysis, was approximately 12% (range 0–40%). Table II summarises the data from these cultures.

Neither the provision of additional feeder cells at 2 weeks post cloning, nor the use of round-bottomed 96 well plates seeded with 5×10^4 feeder

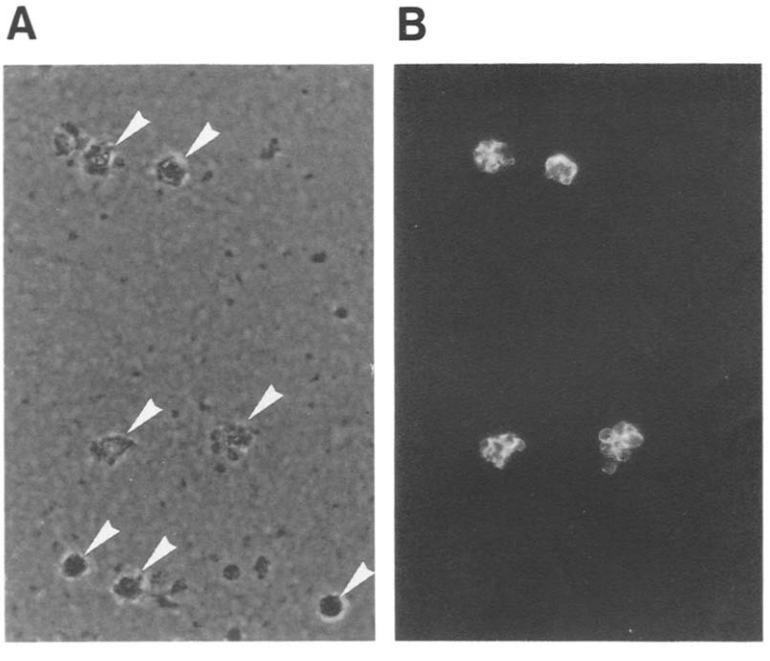

Fig. 2. *A*: phase contrast photograph of a dried, unfixed, thick film of MVF1-infected marmoset red cells. Arrowheads indicate malaria parasites. *B*: same field as shown in *A* showing immunofluorescent staining with the supernatant from a monoclonal, EBV-transformed, marmoset, anti-malaria antibody-secreting LCL (line T3-223-4, see Fig. 1, track 7 for specificity). Note that this antibody recognises only merogonic buds and merozoites within schizont-infected red cells. Bar = 20 μm.

cells/well, improved cloning efficiencies over those of the standard procedure (data not shown).

Characteristics of marmoset LCLS

The majority of EBV-transformed, marmoset lymphoblastoid cells grow in suspension, either singly or in clumps of up to 500 μm in diameter. Cells may adopt a 'hand mirror' shape similar to that of EBV-transformed human lymphocytes. However, marmoset LCLs differ from their human counterparts in that, at each passage, a proportion of the cells attach to the floor of the culture flask, spread and adopt a fibroblastoid morphology.

Provided cell density is kept high ($> 10^5$ cells/ml), established polyclonal and monoclonal marmoset LCLs grow readily in culture. Under optimal conditions, population doubling times as short as 1.5 days are seen. However, twice weekly doubling is suitable for the routine maintenance of lines, with cultures attaining densities of 1.5–3 $\times 10^6$ cells/ml.

EBV-transformed, monoclonal marmoset LCLs are stable in long-term culture. Both growth and immunoglobulin secretion have been maintained over 2 years of continuous culture (to date). The monoclonal lines characterised for this study secrete IgG at levels of up to 12 μg/ml culture supernatant. A small number of IgM-secreting lines have also been generated although these have been less well characterised.

Immunoprecipitation studies demonstrate that marmoset MAbs have been raised against several, distinct, parasite antigens (Fig. 1, tracks 5–7). By immunofluorescence, some of the MAbs localise at, or near, the surface of MVF1 merozoites (Fig. 2). The ontogeny of expression of these antigens during erythrocytic schizogony and their role in eliciting clinical immunity to MVF1 infection in the common marmoset will be described elsewhere.

Discussion

The B lymphocytes of the common marmoset *Callithrix jacchus* can be immortalised by transformation with EBV (Desgranges et al., 1976) and the species is susceptible to infection with the blood stages of several species of malaria parasite, including the line designated MVF1 (Mitchell et al., 1988). By exploiting these two phenomena, we have developed protocols for generating EBV-transformed, monoclonal, LCLs secreting anti-malaria antibodies from marmosets immune to the MVF1 malaria parasite. Although MAbs have been raised from other non-human primates, notably the douroucouli monkey (*Aotus trivirgatus*) (Stanley and Reese, 1985) and the chimpanzee (*Pan troglodytes*) (Van Meel et al., 1985; Van Meurs and Jonker, 1986; Unoki, 1987), we believe this to be the first time that they have been generated from the common marmoset.

EBV isolated from both the B-95-8 and M81 cell lines successfully transformed both PBLs from naive marmosets and splenic lymphocytes from MVF1-infected animals (cf. Ishida and Yamamoto, 1987). Preliminary evidence suggests that it is considerably more difficult to transform the PBLs of older animals (> 3.5 years) (data not shown). The reason for this is unclear although a similar phenomenon has been reported in man (Henderson et al., 1977).

The loss of anti-malaria antibody from later passages of primary cultures may have resulted from overgrowth by populations with other specificities and/or by non-producer cells (Crawford, 1986). Approximately 50% of human lymphocytes polyclonally-activated by EBV in vitro fail to immortalise (Tosato et al., 1985) and transformation, as judged by the onset of significant population expansion, is not apparent until several weeks after exposure of marmoset lymphocytes to the virus. Consequently, it is necessary to compromise between cloning late so that a significant proportion of the population seeded will consist of immortalised cells and cloning early so that the desired specificities will not be lost through overgrowth. The relatively small numbers of anti-malaria antibody-secreting LCLs which were generated from subcultured primary cultures and, in particular, the failure to establish lines from cultures seeded at the equivalent of one or ten cells per well at 16 days PT may reflect this dilemma.

Fresh marmoset PBLs were not available in sufficient numbers to be used as feeder cells for cloning but X-irradiated, EBV-transformed LCLs derived from normal marmoset PBLs proved to be

effective, suggesting that the growth of marmoset LCLs may be subject to autocrine stimulation (see also Tiebout et al., 1987). This would be consistent with the observation of optimal growth at high cell densities. The ability to attach to, and spread on, tissue culture-treated surfaces is a characteristic of EBV-transformed *Callithrix* cells (Desgranges et al., 1976) and does not indicate contamination of LCLs with another cell type. EBV-transformed monoclonal, marmoset LCLs are stable, with both growth and antibody secretion maintained over many months of continuous culture.

In the human system, the majority of clones generated from EBV-transformed PBLs secrete IgM (see, for example, Brown and Miller, 1982; Tosato et al., 1985; Chan et al., 1986). The predominance of IgG-secreting clones detected in this study may reflect the omission from culture medium of 2-mercaptoethanol which is believed to enhance the survival of IgM-secreting, human LCLs and is often added (C. Desgranges, personal communication), but which inhibited the growth of marmoset LCLs (data not shown) and so was not used. However, this result is thought more likely to be artefactual, resulting from a greater affinity of the anti-human IgG used in screening supernatants for anti-malaria antibody for marmoset IgG than that of the anti-human IgM used for marmoset IgM, thereby biasing the assay towards detection of IgG antibodies. Differences in susceptibility to transformation with EBV, both between marmoset and human B lymphocytes and between splenic and peripheral blood B lymphocytes, may also influence the ratio of IgG secretors to IgM secretors generated by these techniques.

Although the methods described in this paper are less efficient than conventional rodent hybridoma technology, the marmoset MAbs so produced possess specificities which derive from a protective immune response and will greatly facilitate investigation of the immunobiology of the *C. jacchus*/MVF1 model system (Mitchell et al., 1988).

As a laboratory primate for immunological study, the common marmoset possesses several useful characteristics: it is small, easy to handle, requires relatively simple husbandry and breeds readily in captivity, usually bearing twins which are haematopoietic chimaeras. Furthermore, as described above, the generation of marmoset MAbs is now feasible. *C. jacchus* is susceptible to infection with diverse human pathogens of major clinical importance including Coronavirus (Russell et al., 1985), Junin virus (Avila et al., 1987), Varicella Zoster virus (Provost et al., 1987), Parainfluenza type 1 (Sendai) Virus (Sunderland et al., 1986), HTLV type 1 (Kinoshita et al., 1985), EBV (Wedderburn et al., 1984), *Campylobacter fetus* (Russell et al., 1985), *Legionella pneumophila* (Baskerville et al., 1983a), *Bordetella bronchiseptica* (Baskerville et al., 1983b), *Chlamydia trachomatis* (Johnson et al., 1981), *Mycoplasma genitalum* (Taylor-Robinson et al., 1987), *Trypanosoma cruzi* (Scott et al., 1985), *Plasmodium vivax* (Mitchell et al., 1988), *Plasmodium brasilianum* (simian form of *P. malariae*) (Wedderburn et al., 1985) and, possibly, HIV 1 (McClure et al., 1987). The protocols detailed in this communication may, therefore, find application in the study of a wide range of human disease.

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