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Demonstration *in vitro* of cell mediated immunity to Epstein–Barr virus in cotton-top tamarins

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SUMMARY

In the course of developing an effective Epstein–Barr (EB) virus vaccine, the immune responses in cotton-top tamarins to a tumourigenic dose of EB virus were studied. Cell mediated responses were measured using a tissue culture 'growth inhibition' assay where peripheral blood lymphocytes were tested for their ability to inhibit the outgrowth of autologous EB virus transformed lymphoblastoid cells. This system has previously been recognized as a very sensitive assay for detecting cell-mediated responses to EB virus in man. Using this assay no cell-mediated immunity was detected up to the time of death in two tamarins following injection with a tumourigenic dose of EB virus. However, two other animals which had recovered from tumours induced by a first dose of EB virus 18 months previously when subsequently re-stimulated with a second tumourigenic dose did exhibit cell-mediated responses. These latter animals remained healthy following the re-challenge and did not show evidence of EB virus-induced disease.

Keywords Epstein–Barr virus cell mediated immunity cotton-top tamarins growth inhibition assay

INTRODUCTION

The cotton-top tamarin, *Saguinus oedipus oedipus*, is the only animal species which consistently gives lesions after experimental infection with the Epstein–Barr (EB) virus (Miller *et al.*, 1977). The species is therefore essential as an animal model in work to develop an EB virus vaccine (Epstein *et al.*, 1985). Previous studies have established a standard large dose of EB virus which gives rise to multiple EB virus genome-positive malignant B cell tumours within 14 to 21 days in all inoculated tamarins (Cleary *et al.*, 1985). In the majority of cases the disease is fatal; in the surviving animals the tumours regress over a period of 8–14 weeks. The tumours are oligo- or monoclonal in origin and resemble the EB virus-associated lymphomas which occur with increased frequency in human organ transplant patients (Cleary *et al.*, 1985).

In man, primary EB virus infection leads to the establishment of a life-long virus carrier state (Nilsson *et al.*, 1971) and the presence in the serum of antibodies to a number of EB viral antigens, some of which (i.e. anti-viral capsid antigen (VCA), anti-EB nuclear antigen (EBNA) and anti-membrane antigen (MA)) are maintained at constant levels throughout life (Henle & Henle, 1979). Recent investigations have demonstrated that cell mediated immunity to EB virus is essential in addition to humoral immunity, and a most important component of this has been shown to be an HLA-restricted T cell-mediated response (Rickinson, 1986). Like humoral immunity to EB virus this cell-mediated immunity is maintained at constant levels throughout life in seropositive individuals. EB virus-specific cytotoxic T cells have been demonstrated both by *in vitro* chromium release assays and by growth inhibition of autologous EB virus-transformed lymphoblastoid cell lines (LCLS) in microtest plate tissue cultures (Rickinson *et al.*, 1981).

In contrast to EB virus infection in man, little is known about the immunological responses in tamarins following EB virus infection. The present experiments aim to examine further the nature of EB virus infection in tamarins. As it is now known that in man cell-mediated immunity is a key host defence mechanism against EB virus, the main aim of the present work has been to investigate cell mediated responses, especially since an understanding of these responses in the tamarin is essential for the development of EB virus vaccines.

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MATERIALS AND METHODS

EB virus infection of cotton-top tamarins

Adult tamarins were obtained from a successful breeding colony (Kirkwood, Epstein & Terlecki, 1983). The animals were injected with a standard 100% tumourigenic dose of EB virus, prepared from B95-8 cells, containing $10^{5.3}$ lymphocyte transforming units (Cleary *et al.*, 1985). The virus was prepared and administered exactly as in earlier work (Epstein *et al.*, 1986).

Cell culture
All cells were cultured in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin. All the cultures were grown at 37°C in a humidified atmosphere of 5% CO_2 in air. Established cell lines were fed twice weekly and cells in microtest plates were fed weekly.

Establishment of virus transformed lymphoblastoid cell lines (LCLS) from tamarin lymphocytes
Unfractionated mononuclear (UM) tamarin cells were separated by standard techniques (Böyum, 1968) from 1 ml samples of peripheral heparinized blood obtained by venepuncture. The UM cells were infected with high titre EB virus prepared from B95-8 cells induced with 20 ng/ml 12-0-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma Chemical Co., Poole, Dorset, UK) and were plated out in flat-bottomed microtest plates (Nunc) at serial dilutions ranging from 10^6 cells/ml to 6×10^4 cells/ml. Foci of growing cells were subcultured firstly into 2 ml Linbro wells (Flow Laboratories Limited, Rickmansworth, England) and then into 25 cm^2 plastic tissue culture flasks (Corning).

Growth inhibition assay
Target cells from the relevant autologous EB virus transformed LCL were seeded into microtest plate *U*-wells (Sterilin Limited, Feltham, England) at serial dilutions ranging from $2{\cdot}5 \times 10^4$ to $1{\cdot}7 \times 10^2$ cells per 0·2 ml well. Twelve replicate wells were seeded per dilution, and of these six received 2×10^4 UM cells freshly prepared from an autologous tamarin peripheral blood sample. The cultures were observed for 4 weeks to assess the incidence of successful target cell outgrowth. Wells were observed using an inverted microscope and scored positive when characteristic progressively growing foci of the target LCLS were present. The effect of the UM cell addition was expressed as the minimum number of autologous LCL cells per well required for a 50% incidence of successful outgrowth in the presence of 2×10^4 UM cells as calculated by the method of Reed & Muench (1938), (Rickinson *et al.*, 1981).

Serology
Antibody levels to EB viral capsid antigen (VCA) were measured by indirect immunofluorescence by standard techniques (Henle & Henle, 1966).

Experimental procedure
In the first set of experiments, two normal tamarins, T1 and T2, were checked for the absence of EB virus antibodies and were then injected with a standard tumourigenic dose of EB virus containing $10^{5{\cdot}3}$ lymphocyte-transforming units (Cleary *et al.*, 1985). The tamarins were regularly monitored clinically for palpable tumours and blood samples were taken on the day of injection (pre-bleed), at 3 weeks after injection, 5 weeks after injection (T2 only) and on the day of death. UM cells were prepared from the peripheral blood and tested on the same day for their ability to inhibit the outgrowth of autologous EB virus-transformed LCL cells in the growth inhibition assay. UM cells prepared from the peripheral blood of a human seropositive donor were tested at the same time against autologous human target cells in an equivalent growth inhibition assay, as a control for the assay. IgG antibodies to VCA were measured in the same blood samples.

Table 1. Summary of the first set of experiments: analysis of humoral and cell-mediated responses in seronegative tamarins (T1 and T2) following a challenge dose of EB virus, with reference to a human seropositive control

	Titre of IgG antibodies to VCA	*In vitro* growth inhibition	Clinical observations presence of lesions
Human seropositive control (SF)	1/512	+	–
T1 Pre-bleed	Negative	ND	None
21 days p.i.	1/64	None	Enlarged lymph nodes
27 days p.i.	1/64	None	Sick with multiple lesions; Submitted to euthanasia
T2 Pre-bleed	Negative	ND	None
21 days p.i.	1/128	None	Enlarged lymph nodes
33 days p.i.	1/128	None	Enlarged lymph nodes
50 days p.i.	1/128	None	Sick with large abdominal mass; Submitted to euthanasia

ND, not done.
p.i., post injection.

In the second set of experiments two tamarins, T3 and T4, which had recovered from tumours induced by a standard challenge dose of EB virus given 18 months previously were inoculated with a further standard tumourigenic dose of EB virus. These tamarins were likewise regularly monitored clinically and blood samples were taken on the day of injection (pre-bleed) and at weeks 1, 2, 4, 6, 8 and 10 after challenge. Humoral and cell-mediated responses were investigated as above.

RESULTS

Establishment of tamarin LCLS
Tamarin lymphocytes appeared to be less susceptible to transformation by EB virus than human lymphocytes since outgrowth of EB virus transformed foci of tamarin lymphocytes occurred after about 3 weeks of culture in comparison to about 1 week for human lymphocytes in the same conditions. This observation is in agreement with previous work by Miller *et al.* (1972).

Response of normal tamarins to EB virus
Table 1 shows a summary of the results obtained in the first set of experiments. Both tamarins developed characteristic lymphoma on challenge with the tumourigenic dose of EB virus as described in earlier work (Cleary *et al.*, 1985); T1 became very poorly and was submitted to euthanasia on ethical grounds on day 27 and T2 also required this treatment on day 50. Both animals developed IgG antibodies to VCA; T1 had a titre of 1/64 and T2 a titre of 1/128. However, no cell mediated responses were detected by the *in vitro* growth inhibition assay up to the time of death.

Table 2 shows a representative growth inhibition assay. UM cells from a seropositive human donor (SF) known to exhibit a cell-mediated response to EB virus were tested for their ability to inhibit the outgrowth of the autologous LCL in exactly the same way as were UM cells from both tamarins 21 days after EB virus

Table 2. Representative experiment showing incidence of successful outgrowth after 4 weeks of EB virus-transformed target cells (LCLS) seeded in the presence and absence of autologous blood mononuclear (UM) cells

Number of target cells seeded per U-well	Seropositive human donor SF		Tamarin T1 21 days p.i.		Tamarin T2 21 days p.i.	
	LCL alone	LCL+2×10⁴UM	LCL alone	LCL+2×10⁴UM	LCL alone	LCL+2×10⁴UM
$2{\cdot}5\times10^4$	6/6	6/6	6/6	6/6	6/6	6/6
$1{\cdot}25\times10^4$	6/6	6/6	6/6	6/6	6/6	6/6
6×10^3	6/6	3/6	6/6	6/6	6/6	6/6
3×10^3	6/6	0/6	6/6	6/6	6/6	6/6
$1{\cdot}5\times10^3$	6/6	0/6	6/6	6/6	6/6	6/6
7×10^2	6/6	0/6	6/6	6/6	6/6	6/6
$3{\cdot}5\times10^2$	6/6	0/6	4/6	6/6	6/6	6/6
$1{\cdot}7\times10^2$	6/6	0/6	3/6	6/6	1/6	5/6

p.i., post injection.

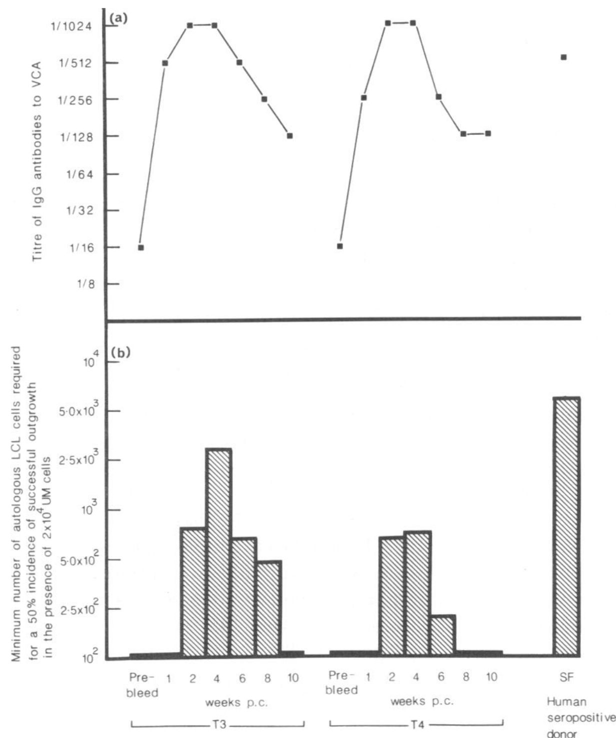

Fig. 1. Ability of UM cells, prepared from successive bleeds of tamarins in the second set of experiments, to inhibit the outgrowth of autologous LCLS: with reference to UM cells prepared from human seropositive control. Titres of IgG antibodies to VCA are also shown. (▧) Minimum number of autologous LCL cells required for a 50% incidence of successful outgrowth in the presence of 2×10^4 UM cells; (■) Titre of IgG antibodies to VCA. p.c. post challenge.

injection. After 4 weeks the outgrowth of the autologous LCL was inhibited by the addition of UM cells from the seropositive donor SF. The minimum number of autologous LCL cells required for a 50% incidence of successful outgrowth in the presence of UM cells being 6×10^3 (Fig. 1) (Reed & Muench, 1938). In contrast, the outgrowth of the tamarin LCLS was not inhibited by autologous UM cells. In fact the addition of these tamarin UM cells appeared to facilitate the outgrowth of the autologous LCL; a similar result has been previously reported in the case of seronegative human donors (Rickinson *et al.*, 1981) probably through a 'feeder' effect. An additional factor in the present work is that the tamarin LCLS are high producers of EB virus so some transformation of B cells within the UM population may have occurred.

Response of previously infected tamarins to EB virus

In this second set of experiments the two tamarins, T3 and T4, which had recovered from tumours induced by a standard tumourigenic dose of EB virus given 18 months previously had an anti-VCA titre of 1/16 but no cell mediated immunity was detected by the *in vitro* growth inhibition assay. On challenge with a further tumourigenic dose of EB virus, no enlarged lymph nodes nor lymphoma developed, in contrast to the seronegative tamarins. The humoral and cell mediated responses shown by these tamarins after the challenge are illustrated in Fig. 1.

The anti-VCA titres rose from 1/16 at the beginning of the experiment to 1/1000 in both tamarins at 2 and 4 weeks after challenge and thereafter declined. Cell-mediated responses were detected by the *in vitro* growth inhibition assay using autologous LCLS at 2 weeks after challenge in both animals. The peak of the response was seen at 4 weeks and the response then declined so that by 10 weeks the cell-mediated response could no longer be detected in either animal. *In vitro* growth inhibition was not observed when heterologous EB virus transformed tamarin lymphocytes were used as target cells.

DISCUSSION

As cotton-top tamarins are rare and costly animals only small numbers can be used for experimental work with EB virus, as has previously been explained (Epstein *et al.*, 1986). In the present work, the two seronegative tamarins, T1 and T2, developed moderate levels of antibody to VCA within 3 weeks of injection with EB virus. In a previous study by other workers low levels of antibodies to VCA were not detected until at least 6

weeks after injection with EB virus (Johnson *et al.*, 1983). However, in the latter investigation fractionally less virus (10^5 transforming units in 1 ml) was injected. The tamarins, T3 and T4, which had recovered from tumours induced 18 months earlier by a tumourigenic dose of EB virus, had only low antibody levels to VCA. Their antibody titres of 1 in 16 are lower than that normally observed in healthy human seropositive serum (Yao, Rickinson & Epstein, 1985).

The present work demonstrates that tamarins are capable of developing a cell-mediated response to EB virus. This response was observed in previously infected animals which were restimulated *in vivo* with a second dose of EB virus. In contrast, tamarins recently infected with EB virus did not exhibit cell-mediated immunity as detected in the growth inhibition assay up to the time of death (27 and 50 days after EB virus injection). In this connection it is of interest that no cell mediated regression of EB virus infected autologous B cells is normally observed within the first few months in patients suffering from acute infectious mononucleosis (Rickinson *et al.*, 1980).

Studies in man have demonstrated that healthy individuals previously infected with EB virus maintain a life-long virus carrier state since constant moderate antibody titres (usually > 1 in 128) to VCA are present in the serum and virus specific memory T cells continually circulate in the peripheral blood (Yao *et al.*, 1985). This life-long virus carrier state is presumably due to chronic, low-grade replication of the virus at a permissive site in the oro-pharynx or naso-pharynx since EB virus can be detected in throat washings from the vast majority of seropositive human subjects (Yao *et al.*, 1985). The present findings that previously infected tamarins not only have low antibody levels to VCA but also that their peripheral blood lymphocytes are not able to inhibit the outgrowth of autologous LCLS indicates that tamarins do not maintain a life-long EB virus carrier state in exactly the same way as is observed in man.

However, cell mediated responses were demonstrated in the previously infected tamarins within 2 weeks of restimulation with a further dose of EB virus. This indicated the presence in these animals of a population of memory T cells which were expanded on restimulation with antigen *in vivo*. Since these tamarins did not succumb to EB virus induced disease on re-challenge it appears that both humoral and cell-mediated immunity are important factors in conferring this protection. It is obviously important to investigate whether a similer cell-mediated response is observed in rare tamarins like T3 and T4 as they recover from their initial EB virus infection. This question will be addressed as soon as such animals become available.

The target antigens recognized by this immune response are open to speculation. The tamarin LCL target cells express the range of EB virus latent antigens and a percentage of the cells express late gene products (Finerty *et al.*, 1988). T cells recognize antigens as processed peptides and any of these latent, early or late EB viral antigens could function as targets for this immune response as has been recently reviewed by Wallace & Murray (1988).

Additional evidence for the importance of cell-mediated immunity in the tamarin has come from recent experiments which demonstrate that vaccinated animals can be protected from EB virus challenge in the absence of circulating EB virus neutralizing antibody (Morgan *et al.*, 1988). Further characterization of cell-mediated immunity to the range of EB virus antigens in the tamarin is clearly important for the development of EB virus vaccines.

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REFERENCES

Böyum, A. (1968) Separation of leucocytes from blood and bone marrow. *Scand. J. clin. Lab. Invest.* **21**, Suppl. **97**, 77.

Cleary, M.L., Epstein, M.A., Finerty, S., Dorfman, R.F., Bornkamm, G.W., Kirkwood, J.K., Morgan, A.J. & Sklar, J. (1985) Individual tumors of multifocal EB virus-induced malignant lymphomas in tamarins arise from different B-cell clones. *Science* **228**, 722.

Epstein, M.A., Morgan, A.J., Finerty, S., Randle, B.J. & Kirkwood, J.K. (1985) Protection of cotton-top tamarins against EB virus-induced malignant lymphoma by a prototype subunit vaccine. *Nature (London)* **318**, 287.

Epstein, M.A., Randle, B.J., Finerty, S. & Kirkwood, J.K. (1986) Not all potently neutralizing vaccine-induced antibodies to Epstein–Barr virus ensure protection of susceptible experimental animals. *Clin. exp. Immunol.* **63**, 485.

Finerty, S., Young, L.S., Scullion, F.T., Morgan, A.J. & Rickinson, A.B. (1988) Epstein-Barr virus gene expression in lymphomas induced by the virus in cotton-top tamarins. (In preparation).

Henle, G. & Henle, W. (1966) Immunofluorescence in cells derived from Burkitt's lymphoma. *J. Bact.* **91**, 1248.

Henle, G. & Henle, W. (1979) The virus as the etiologic agent of infectious mononucleosis. In: *The Epstein–Barr Virus* (Ed. by M.A. Epstein & B.G. Achong) p. 297. Springer-Verlag, Berlin, Heidelberg, New York.

Johnson, D.R., Wolfe, L.G., Levan, G., Klein, G., Ernberg, I. & Aman, P. (1983) Epstein–Barr virus (EBV)-induced lymphoproliferative disease in cotton topped marmosets. *Int. J. Cancer* **31**, 91.

Kirkwood, J.K., Epstein, M.A. & Terlecki, A.J. (1983) Factors influencing population growth of a colony of cotton-top tamarins. *Lab. Anim.* **17**, 35.

Miller, G., Shope, T., Coope, D., Waters, L., Pagano, J., Bornkamm, G.W. & Henle, W. (1977) Lymphoma in cotton-top marmosets after inoculation with Epstein–Barr virus: tumor incidence, histologic spectrum antibody responses, demonstration of viral DNA, and characterization of viruses. *J. exp. Med.* **145**, 948.

Miller, G., Shope, T., Lisco, H., Stitt, D. & Lipman, M. (1972) Epstein-Barr virus: transformation, cytopathic changes, and viral antigens in squirrel monkey and marmoset leukocytes. *Proc. natn Acad. Sci. USA* **69**, 383.

Morgan, A.J., Mackett, M., Finerty, S., Arrand, J.R., Scullion, F. & Epstein, M.A. (1988) Recombinant vaccinia virus expressing Epstein–Barr virus glycoprotein gp340 protects cottontop tamarins against EB virus-induced malignant lymphomas. *J. med. Virol.* (in press).

Nilsson, K., Klein, G., Henle, W. & Henle, G. (1971) The establishment of lymphoblastoid cell lines from adult and foetal human lymphoid tissue and its dependence on EBV. *Int. J. Cancer* **8**, 443.

Reed, L.J. & Muench, H. (1938) A simple method of estimating fifty per cent end points. *Amer. J. Hyg.* **27**, 493.

Rickinson, A.B. (1986) Cellular immunological responses to the virus infection. In: *The Epstein–Barr Virus: Recent Advances* (Ed. by M.A. Epstein & B.G. Achong) p. 75. Heinemann, London.

Rickinson, A.B., Moss, D.J., Allen, D.J., Wallace, L.E., Rowe, M. & Epstein, M.A. (1981) Reactivation of Epstein–Barr virus-specific

cytotoxic T cells by *in vitro* stimulation with the autologous lymphoblastoid cell line. *Int. J. Cancer* **27,** 593.

RICKINSON, A.B., MOSS, D.J., POPE, J.H. & AHLBERG, N. (1980) Long-term T-cell-mediated immunity to Epstein–Barr virus in man. IV. Development of T-cell memory in convalescent infectious mononucleosis patients. *Int. J. Cancer* **25,** 59.

WALLACE, L.E. & MURRAY, R.J. (1988) Immunological aspects of the EBV system. *Adv. in Viral Oncology*, **8,** (in press).

YAO, Q.Y., RICKINSON, A.B. & EPSTEIN, M.A. (1985) A re-examination of the Epstein–Barr virus carrier state in healthy seropositive individuals. *Int. J. Cancer* **35,** 35.