Expression of Virus-specific, Thymus-specific and Tumour-specific Antigens in Lymphoblastoid Cell Lines Derived from Marek's Disease Lymphomas

By L. J. N. ROSS, P. C. POWELL, D. J. WALKER, M. RENNIE AND L. N. PAYNE

Houghton Poultry Research Station, Houghton, Huntingdon, Cambs., PE17 2DA

(Accepted 26 November 1976)

SUMMARY

The expression of virus, thymus and tumour-specific antigens was studied in two Marek's disease lymphoblastoid cell lines (MSB-I and HPRS-line 2).

The proportions of cells which spontaneously expressed virus antigens or which formed infective centres in vitro were considerably higher in MSB-I than in HPRS-line 2, but did not exceed 10%. In contrast to virus antigens, thymus-specific and tumour-specific antigens were expressed on the majority of the cells. Treatment with IdUrd increased the proportion of cells forming infective centres in both cell lines and the proportion of cells expressing virus antigens in MSB-I. A decline in the proportion of cells forming infective centres in IdUrd-treated and in untreated cultures was noted during continuous subculture of both cell lines.

Direct evidence for the presence of virus-specific antigens in MSB-I cells was obtained by immunodiffusion. The results suggested further that lymphoblastoid cells are unable to synthesize a major precipitating glycoprotein antigen (A antigen) normally associated with infection of permissive cells with MDV.

Analysis of surface proteins of normal thymus cells labelled by lactoperoxidase-catalysed iodination showed that thymus-specific determinants are associated with iodinated polypeptides in the mol. wt. range 45,000 to 47,000, 50,000 to 58,000 and 90,000 to 150,000. The major thymus-specific polypeptide exposed at the surface of normal thymus cells was in the mol. wt. range of 50,000 to 58,000. Surface proteins of lymphoblastoid cells were not accessible to iodination.

INTRODUCTION

The predominance of thymus-derived lymphocytes in Marek's disease tumours (Hudson & Payne, 1973; Rouse, Wells & Warner, 1973) and the establishment from these tumours of a number of cell lines bearing thymus-specific and tumour-specific antigens (Akiyama, Kato & Iwa, 1973; Powell et al. 1974; Witter et al. 1975) suggest that thymus-derived cells are neoplastically transformed in Marek's disease. The role of the virus genome and of virus-specific products in the establishment and maintenance of the transformed state is not known. However, it has been reported that a small proportion of the cells of the lymphoblastoid cell lines MSB-1 and HPRS-line 2 express MDV-specific antigens as shown by immunofluorescence (Akiyama et al. 1973; Powell et al. 1974) and it has been
estimated that MSB-1 cells contain on average 60 to 90 genome equivalents of MDV DNA per cell (Nazerian & Lee, 1974).

In this study, we have compared the expression of MDV-specific antigens, thymus-specific antigens and tumour-specific antigens in the two cell lines MSB-1 and HPRS-line 2 to further our understanding of virus-cell relationships in MDV transformed cells. Specifically, we have investigated (1) whether spontaneous and IdUrd induced expression of MDV changes during continuous growth and whether the two cell lines behaved similarly in these respects, (2) whether virus-specific antigens present in cell lines are identical to precipitating antigens previously characterized as MDV-specific and whether there is any evidence of restricted expression of some antigens in the cell lines and (3) whether thymus-specific antigens normally present at the surface of thymus cells are also present in the cell lines.

Our results show that the proportion of cells containing infectious virus declined during continuous growth in vitro and differed markedly in the two cell lines even following activation with IdUrd. We have shown further that host-determined factors control the expression of A antigen in the MSB-1 cell line, that virus and tumour-specific antigens are serologically distinct and that cell surface proteins carrying thymus-specific determinants are modified in the cell lines.

METHODS

Virus and infective centre assay. Strain HPRS-16 (Purchase & Biggs, 1967) of Marek’s disease virus (MDV) and its attenuated derivative HPRS-16/att (Churchill, Chubb & Baxendale, 1969) grown in chick embryo fibroblasts were used as cell-associated viruses. Strain FC126 of herpesvirus of turkeys (Witter et al. 1970) was also propagated as cell-associated virus. Infectivity was estimated by plating known numbers of cells on monolayers of chicken kidney cells in 50 mm dishes as described by Churchill (1968). Plaques were counted after 6 days at 37 °C. Infectivity of lymphoblastoid cell lines was determined by plating 1 to 2 × 10⁶ cells/dish. Unattached cells were removed after 24 h and the medium replaced.

Cells. Chick embryo fibroblasts (CEF) were obtained from 10- to 12-day-old embryos from the HPRS flock of Rhode Island Red (RIR) chickens, free from MDV and avian leukosis virus of subgroups A, B and C.

Chicken kidney cells (CKC) were obtained from 10- to 30-day-old RIR birds kept in isolators, and monolayers were prepared as described by Churchill (1968).

Lymphoblastoid cell lines. Cells employed were obtained from established lines maintained at Houghton Poultry Research Station. MSB-1 cell line, derived from the spleen of an MDV-infected bird, was kindly supplied by Dr Kato, Osaka and was used 4 months after initiation of the culture. HPRS-line 2 cells (Powell et al. 1974) established from ovarian tumours of RIR birds, were used 19 months after initiating the cultures. Growth medium was RPMI medium (Flow Laboratories) supplemented with 10% foetal calf serum (FCS), 10% tryptose phosphate broth (TPB) and antibiotics.

Single cell suspensions of normal thymus cells (T cells) were obtained by teasing thymus tissue derived from neonatally bursectomized and X-irradiated RIR birds (Payne & Rennie, 1970) in RPMI growth medium. Peripheral blood cells (PBL) were also obtained from these birds.

Cultivation of lymphoblastoid cell lines. Cell lines were cultured in roller bottles. This new method of growing the cells yielded 2-5 times higher concentrations of viable cells than those reported in static culture conditions (Kato & Akiyama, 1975; Powell et al.
Antigen expression in MD cell lines

1975) and also substantially increased the proportion of viable cells. Cultures were initiated by seeding 2 l roller bottles containing 200 ml of RPMI growth medium at a final concentration of $0.25 \times 10^8$ cells/ml and were rolled at 0.15 rev/min at 37 °C. The concentration of viable cells increased nine- to tenfold on average after 4 days and usually declined thereafter unless subcultured. Subculture was effected by transferring 50 ml of the cell suspension to a new bottle containing 150 ml of fresh medium. Using this procedure, $5 \times 10^8$ viable cells were readily obtained from each roller bottle every 4 days.

Antisera. Sera collected from a group of leukosis-free Brown Leghorns which had survived natural exposure to MDV were pooled and used as a source of antibody to MDV-specific antigens (a-MDV ser.). This serum is referred to as convalescent serum.

The preparation of antiserum against thymus-derived lymphocytes has been described (Payne, Powell & Rennie, 1974). Rabbits were immunized with $10^9$ thymus cells given intravenously twice at intervals of 14 days. Sera were collected 7 days after the second injection and were absorbed with chicken liver homogenates, chicken erythrocytes, insolubilized chicken immunoglobulin (Ig) and bursa (B) cells.

The preparation of antiserum to B cells (anti-B serum) has been described (Payne et al. 1974).

Both anti-B and anti-thymus sera were shown to be free from residual activity against materials used for absorption using immunofluorescence tests (Payne et al. 1974).

Anti-tumour antibody was raised by immunizing adult rabbits intravenously (i.v.) twice with $10^9$ MD lymphoma cells given 14 days apart. Serum was collected 7 days after the second inoculation and was absorbed 2 to 4 times with chicken-derived liver cells, erythrocytes, normal thymus cells, B cells, spleen cells and PBL using 1 vol. packed cells of each type for 2 vol. of serum.

Swine anti-rabbit 7S serum and rabbit anti-chick Ig were purchased from Nordic Diagnostics Ltd. Igs were also isolated by precipitation with ammonium sulphate and purified by Sephadex G 200 chromatography.

Antibody to the Bryan standard strain of Rous sarcoma virus (BS-RSV) was raised by hyperimmunization of susceptible Brown Leghorns. The serum had high neutralizing activity for leucosis and sarcoma viruses of the A subgroup.

Preparation of iodinated antibody. Purified immunoglobins were iodinated using 1 mCi of carrier-free Na$^{131}$I (Radiochemical Centre, Amersham) and 100 µg protein in the presence of 50 µg chloramine T. The volume was adjusted to 50 µl with phosphate buffer, pH 7.3, at a final molarity of 0.2 M. The reaction was terminated after 2 min at room temperature by addition of 20 µl of 0.4 mg/ml of tyrosine. Free iodine, iodide and labelled tyrosine were removed by passage through a Sephadex G-25 column equilibrated with PBS containing 2 % FCS. The iodinated Igs were further purified by affinity chromatography using insolubilized normal rabbit serum or chick serum (10 ml/ml of iodinated Ig. The sp. act. of the purified Ig was approx. $1.3 \times 10^5$ ct/min/µg assuming complete recovery of protein.

Radiolabelling of cells. External proteins were iodinated using the lactoperoxidase-catalysed method essentially as described by Hynes (1973). Viable cells were obtained from actively growing lymphoblastoid cells by centrifugation through Ficoll-hypaque. Normal thymus cells were prepared in a similar way from cell suspensions which had been incubated in growth medium for 2 h at 37 °C. Cells were washed three times in PBS and were resuspended in PBS containing 50 mM-D-glucose (5 $\times 10^7$ cells/0.1 ml). Carrier-free Na$^{131}$I was added (300 µCi/0.1 ml) followed by 0.1 ml samples of lactoperoxidase (0.25 mg/ml, Sigma), and glucose oxidase (1 unit/ml, Sigma). The reaction was allowed
to proceed for 10 min at room temperature and was terminated by addition of 10 ml of PBS containing 50 mM-KI, 2 mM-phenyl methyl sulphonyl fluoride (PSF) and 25 mM-sodium azide. Cells were washed twice in PBS containing PSF and azide and were stored at -70 °C until solubilized.

**Extraction and solubilization of membrane constituents.** In previous experiments (Ross, 1974) membrane glycoproteins were extracted using 0.5 % Nonidet P40 (Shell Ltd) for 10 min at 4 °C, followed by centrifugation at 5000 g for 10 min. It was found subsequently that a large proportion of labelled glycoproteins in the 5000 g supernatant was sedimented at 10000 g and had not been solubilized. In the present study, membrane materials were extracted with 0.5 % NP40 for 10 min at 4 °C followed by centrifugation at 3000 g for 10 min to sediment cells. The supernatant was solubilized by adding deoxycholate to a final concentration of 0.5 % (w/v) and centrifuging at 10000 g for 1 h. The final supernatant contained over 90 % of 125I counts associated originally with surface proteins labelled by lactoperoxidase-catalysed iodination.

**Immunoprecipitation.** Indirect immunoprecipitation was carried out as follows. Solubilized material (0.1 ml) was incubated with an equal volume of antiserum for 1 h at 37 °C and then at 4 °C overnight. The resulting precipitate was sedimented by centrifugation at 10000 g for 20 min, resuspended in a small volume of PBS and centrifuged at 10000 g for 20 min through a 3 cm layer of 5 % sucrose in PBS at 4 °C. The sedimented materials were washed once again in PBS before analysis by polyacrylamide gel electrophoresis.

**Polyacrylamide gel electrophoresis.** Electrophoresis was performed in 6 mm x 12 cm cylindrical gels using essentially the discontinuous pH + SDS system described by Dimmock & Watson (1969). Samples for electrophoresis were dissolved by boiling for 1 to 2 min in 2 % SDS + 2 % mercaptoethanol + 0.5 M-urea + 0.06 M-tris-phosphate, pH 6.9 + 10 % sucrose + phenol red. Electrophoresis was carried out at 4 mA/tube and was stopped when phenol red had travelled 6 to 7 cm in the separation gel. Staining of proteins with Coomassie blue and fractionation of gels for estimation of radioactivity have been described (Ross, Biggs & Newton, 1973).

Molecular weights were estimated using the following markers: chymotrypsinogen (25700), ovalbumin (43000), bovine serum albumin (67000) and β-galactosidase (130000).

**Immunodiffusion tests** were carried out in 1 % Difco Noble agar containing 8 % NaCl and 0.02 % azide as described previously (Ross et al. 1975). Partially purified preparations of A, B and C antigens were obtained as previously described (Ross et al. 1975).

**Immunofluorescence tests** were carried out as described previously using convalescent MD serum and FITC-labelled rabbit anti-chicken globulin (Ross et al. 1975). The proportion of cells fluorescing was estimated by examining 500 cells on duplicate slides (total of 1000 cells) for each preparation. Cells treated with normal chicken serum did not stain.

**Antibody binding assay.** Suspensions of 10⁶ cells in 0.1 ml of diluent (PBS containing 2 % FCS and 0.1 % azide) were treated with equal volumes of 1/20 to 1/200 dilutions of immune sera or normal sera for 30 min at 4 °C. Cells were then washed twice with 2 ml of diluent, resuspended in 0.1 ml of diluent and 0.1 ml of iodinated anti-globulin containing approx. 20000 ct/min was added. After incubation for 30 min at 4 °C, unbound labelled globulin was removed by centrifuging the cells through a layer of FCS followed by two further washes using PBS.

Cell bound radioactivity was determined either directly using a gamma counter, 'bulk counting', or by autoradiography. Smears of equal numbers of cells were fixed in methanol, air-dried and dipped in Kodak's K2 liquid emulsion for autoradiography. Grains were developed after 10 days at 4 °C using D19 developer. Grain counts were performed on a
Antigen expression in MD cell lines

minimum of 200 cells per slide and on duplicate slides for each group. The method of Stillström (1963) was used to correct background radioactivity and non-specific binding which was assessed by estimating the grain count in preparations treated with normal sera.

Preliminary experiments using the 'bulk counting' method suggested that uptake of radioactivity was not limited by insufficient amounts of 125I anti-globulin. This was shown by the fact that uptake of radioactivity dropped rapidly upon dilution (1/20 to 1/200) of immune sera used in the first stage of the test. If the amount of labelled anti-globulin used had been a limiting factor, a plateau would have been observed in the uptake of radioactivity.

Results are expressed as ratios of counts taken up using immune serum to counts taken up using normal serum after subtracting background counts.

**Test for Ig receptors.** The presence of Ig receptors at the surface of lymphoblastoid cell lines was examined essentially using the method described by Westmoreland & Watkins (1974), with the exception that reactions were carried out in suspension. Briefly, 10⁶ cells purified by centrifugation through Ficoll-hypaque were incubated with 0.5 ml of 1/20 dilution of rabbit anti-sheep erythrocyte serum (Wellcome) in PBS + 2% FCS for 30 min at 37 °C. Cells were washed twice in PBS + FCS and 1 ml of 0.5% suspension of sheep erythrocytes added and incubated for 30 min at 37 °C. The cell suspensions were then stained with Natt and Herrick's stain (Natt & Herrick, 1952) for differentiation of lymphocytes. The fixative present in the stain prevented lysis of erythrocytes and allowed permanent preparations to be made. Thymus cells and peripheral blood cells derived from bursectomized birds served as controls. These cells were washed in growth medium before use and were purified by centrifugation through Ficoll-hypaque.

**RESULTS**

**Evidence for virus-specific antigens in lymphoblastoid cells**

Direct evidence for the presence of MDV-specific antigens in MSB-1 cells was obtained by immunodiffusion. This was possible since the conditions of culture described here allowed the preparation of extracts of large numbers of cells which were essential for the demonstration of precipitins. At least three precipitating antigens were demonstrated in extracts of MSB-1 cells (5 x 10⁶/ml) using convalescent serum (Fig. 1). These were identical to antigens present in CEF infected with MDV/att which included the B and C antigens described previously (Ross et al. 1975). Complete identity between the 3 precipitins obtained with extracts of MSB-1 cells and those of MDV-infected CEF was shown in further tests using appropriate dilutions of extracts of MDV-infected CEF. All three precipitins are considered to be MDV-specific since serologically identical antigens are produced in chick and in duck cells infected with MDV (Ross et al. 1973, 1975) and are not present in uninfected cells or in chick cells infected with herpes simplex virus. Furthermore, since extracts of HPRS-line 2 (5 x 10⁶ cells/ml to 5 x 10⁷/ml) did not react, it follows that the precipitins observed with MSB-1 cells could not be due to reactions with tumour antigens or thymus antigens which are known to occur in both cell lines.

Extracts of MSB-1 cells and of HPRS-line 2 cells and the materials obtained from the medium of these cultures by precipitation with ammonium sulphate did not contain the 'A' antigen (Churchill et al. 1969). This antigen has been shown to be a major precipitating glycoprotein induced in CEF and DEF cultures infected with some strains of MDV and HVT (Ross et al. 1973, 1975). However, virus isolated from both cell lines by co-cultivation
Fig. 1. Agar gel immunodiffusion test of extracts of MSB-1 cells (cultured 11 months) and HPRS-line 2 cells (cultured 20 months) and of CEF infected with MDV/att against convalescent serum (a-MDV ser). The three precipitins obtained with extracts of MSB-1 cells were identical with those obtained with extracts of CEF cells infected with MDV/att (only 2 lines visible in this photograph). The single precipitin obtained with concentrated culture medium derived from CKC infected with virus isolated from MSB-1 cells (MSB-1 isolate 'A' antigen) is identical to purified A antigen obtained from MDV-infected cultures (MDV A) and from HVT-infected cultures (HVT A).

with CKC was capable of inducing the A antigen when grown in CKC. Fig. 1 shows that purified antigen (MSB-1 isolate A) obtained from CKC infected with virus isolated from MSB-1 cells was serologically identical to reference A antigen (MDV and HVT A) preparations obtained from MDV or HVT infected cultures. Extracts of uninfected CKC did not react in these tests.
Antigen expression in MD cell lines

Further experiments were carried out to determine whether the failure to detect A antigen in MSB-1 cells was due to degradation of the antigen or to failure of synthesis. A purified preparation of A antigen was incubated for 24 h at 37 °C with extracts of MSB-1 cells (10⁶ cells/ml) and also with actively growing MSB-1 cells (2 × 10⁶ cells/ml) and remaining precipitating activity was assessed by dilution to end point of titration by immunodiffusion. No reduction in titre was noted in preparations treated with MSB-1 cells or with normal thymus cells. We concluded therefore that failure to detect A antigen in MSB-1 cells was not attributable to degradation and was thus likely to be due to failure of synthesis.

It is of interest that although extracts of HPRS-line 2 cells which had been grown for 20 months did not form precipitins with convalescent serum, the virus isolated from these cells (a derivative of HPRS-16) had retained the ability to induce A, B and C antigens when grown in CKC (not shown). Pathogenicity tests carried out in RIR chickens showed that virus isolated from HPRS-line 2 after 20 months of continuous subculture was still pathogenic since 5/5-day-old birds inoculated with 10⁴ p.f.u. intraperitoneally developed gross lesions characteristic of MD. None of the 5 birds inoculated with normal CKC developed the disease. This suggests that attenuation of HPRS-16 by serial passage in CKC and the concurrent loss of the A antigen reported earlier (Churchill et al. 1969) is not an inevitable consequence of long term cultivation in vitro, but depends rather on the host cell and perhaps on the nature of the association between virus genome and the host genome.

Activation of MDV in lymphoblastoid cell lines

Activation experiments using IdUrd were carried out to extend our investigation of MDV-cell relationship in cell lines. Preliminary experiments established that optimum conditions for activation judged by infective centre assay were as follows. Cells were cultured to log phase in medium containing dialysed FCS and no TPB. IdUrd was added to a final concentration of 30 μg/ml and incubation continued for 24 h. Normal medium was then added and cells were processed for immunofluorescence 48 h later and were plated on CKC monolayers for infective centres. Cells did not increase in number in the presence of the drug, but viability usually remained high as shown by trypan blue staining and cell division resumed on returning the cells to normal growth conditions.

The proportion of cells containing infectious virus as shown by infective centre assay was approx. 100 times higher in the MSB-1 cell line (Fig. 2b) than in HPRS-line 2 (Fig. 3b) and declined in both cell lines during 6 months of continuous subculture. Treatment with IdUrd enhanced the proportion of cells scoring as infective centres in both cell lines in all experiments but failed to raise it to the levels observed in the first experiment, indicating that a change in the cell population had occurred during continuous growth. The decline in the capacity to form plaques was so marked that by 23 months it became impossible to demonstrate infectious virus in HPRS-line 2 by plating 2 × 10⁶ cells. Furthermore, the plaques which developed from HPRS-line 2 cells following activation with IdUrd were smaller than those obtained with MSB-1 cells.

The proportion of cells expressing antigens detected by immunofluorescence was 10 times higher in MSB-1 cultures (Fig. 2a) than in HPRS-line 2 (Fig. 3a) but did not appear to decline throughout the 6 month period of experiments in contrast to the infective centres. In HPRS-line 2 cells which had been cultured for more than 19 months, fluorescence was faint and diffuse and was confined to the cytoplasm of 0.2 to 0.5% of cells. MSB-1 cells in contrast showed bright fluorescence throughout the cells and granular fluorescence in
Fig. 2. IdUrd activation of MDV in MSB-1 cells. Cells were processed for immunofluorescence and were plated for infective centres 48 h after withdrawing the drug. (a) Proportion of cells synthesizing virus-specific antigens determined by immunofluorescence using convalescent serum, (b) proportion of cells forming plaques; (square) treated with PBS, (circle) treated with IdUrd. The levels of significance for differences between treated and untreated cultures determined by $X^2$ analysis are indicated. Vertical bars in (b) refer to standard error of means of 4 replicate dishes.

Fig. 3. IdUrd activation of MDV in HPRS-line 2 cells. Legend as for Fig. 2.
Antigen expression in MD cell lines

Table 1. Antibody uptake by viable unfixed cells assessed by bulk counting

<table>
<thead>
<tr>
<th>Cells</th>
<th>Anti-MDV</th>
<th>Anti-thymus</th>
<th>Anti-tumour</th>
<th>Anti-B</th>
<th>Anti-RSV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSB-1 (cultured 4 months)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-MDV</td>
<td>2.06 ± 0.34*</td>
<td>2.53 ± 0.07*</td>
<td>2.50 ± 0.1*</td>
<td>NT</td>
<td>1.06 ± 0.04</td>
</tr>
<tr>
<td>Anti-thymus</td>
<td>0.95 ± 0.02</td>
<td>2.28 ± 0.01*</td>
<td>2.3 ± 0.07*</td>
<td>NT</td>
<td>1.08 ± 0.04</td>
</tr>
<tr>
<td>Anti-tumour</td>
<td>2.14 ± 0.02*</td>
<td>2.05 ± 0.02*</td>
<td>2.0 ± 0.08*</td>
<td>NT</td>
<td>0.85 ± 0.1</td>
</tr>
<tr>
<td>Anti-B</td>
<td>0.8 ± 0.05</td>
<td>3.43 ± 0.07*</td>
<td>1.8 ± 0.05*</td>
<td>NT</td>
<td>0.86 ± 0.04</td>
</tr>
<tr>
<td>Anti-RSV</td>
<td>0.88 ± 0.07</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>2.29 ± 0.05*</td>
</tr>
<tr>
<td>Normal thymus</td>
<td>1.03 ± 0.02</td>
<td>2.21 ± 0.05*</td>
<td>2.4 ± 0.1*</td>
<td>NT</td>
<td>1.08 ± 0.08</td>
</tr>
<tr>
<td>Line 2 (cultured 19 months)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-MDV</td>
<td>0.8 ± 0.05</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>2.0 ± 0.05</td>
</tr>
<tr>
<td>Anti-thymus</td>
<td>1.0 ± 0.02</td>
<td>1.02 ± 0.02</td>
<td>NT</td>
<td>NT</td>
<td>0.99 ± 0.08</td>
</tr>
<tr>
<td>Anti-tumour</td>
<td>1.10 ± 0.06</td>
<td>0.92 ± 0.02</td>
<td>1.01 ± 0.02</td>
<td>NT</td>
<td>0.96 ± 0.08</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSB-1 (cultured 20 months)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-MDV</td>
<td>1.17 ± 0.08</td>
<td>1.37 ± 0.08*</td>
<td>2.55 ± 0.16*</td>
<td>1.06 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Anti-thymus</td>
<td>0.75 ± 0.06</td>
<td>1.87 ± 0.14*</td>
<td>1.72 ± 0.01*</td>
<td>0.82 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Anti-tumour</td>
<td>0.87 ± 0.29</td>
<td>2.88 ± 0.3*</td>
<td>1.57 ± 0.19*</td>
<td>1.03 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>Anti-B</td>
<td>2.66 ± 0.08*</td>
<td>0.8 ± 0.1</td>
<td>1.13 ± 0.07</td>
<td>0.72 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Anti-RSV</td>
<td>0.80 ± 0.04</td>
<td>1.12 ± 0.07</td>
<td>1.30 ± 0.03</td>
<td>1.18 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

* Denotes significant reactions of immune serum compared to controls, i.e. ratios greater than the mean ratio for controls by 3 x s.d. The mean ratio and s.d. for controls (0.99 ± 0.1) was calculated from ratios obtained for interaction of normal thymus and CEF cells with anti-B, anti-MDV and anti-sarcoma sera.

† Mean and standard error of ct/min immune serum. Data derived from 4 replicate estimations.

‡ Final dilution of antiserum.

§ Not tested.

the cytoplasm in all experiments over a period of 6 months. Treatment with IdUrd increased the proportion of MSB-1 cells which expressed virus antigens about threefold. This was not observed in HPRS-line 2 cells except in the first experiment.

These results suggested that complete virus genome is present in both cell lines in many more cells than is normally detectable and that virus repression might be stronger in HPRS-line 2 than in MSB-1 cells. However, in view of the considerable difference in the ‘age’ of HPRS-line 2 and of MSB-1 cells used here, it is possible that the differences in virus expression observed between these two cell lines is purely temporal and is the result of selection of non-producer cells during long term growth.

More sensitive and quantifiable tests involving measurement of uptake of iodinated antibody confirmed and extended the immunofluorescence results. Cells were treated with appropriate dilutions of rabbit anti-thymus, anti-tumour (anti-lymphoma) and anti-B antisera and of chicken anti-MDV and anti-RSV antisera as described in Methods. Samples of cells were also treated with normal rabbit serum (1/10) and with normal chick serum (1/20). After removal of unbound serum by washing, 125I-labelled Ig prepared against rabbit 7S Ig or against chick Ig was added as appropriate, using 20 000 ct/min of iodinated antibody for each reaction mixture. The results of two experiments assessed by ‘bulk counting’ are shown in Table 1. Reactions were considered positive if uptake of radioactivity was at least 1.3 times higher when immune serum was used compared to normal serum. This lower limit was shown to exceed the mean obtained for 8 independent controls by 3 standard deviations. On this basis, the results obtained agreed well on the
Fig. 4. Reactivity of (a) anti-MDV serum, (b) anti-thymus serum and (c) anti-tumour serum determined by uptake of $^{125}$I-labelled anti-Ig and autoradiography. Grains/cell and % cells labelled are means of 200 observations from duplicate slides for each preparation and have been corrected for background and non-specific uptake. PBL and T refer to peripheral blood and to thymus cells derived from normal bursectomized birds respectively. CEF and MDV/CEF refer to chick embryo fibroblasts and to chick embryo fibroblasts infected with MDV respectively. MSB and L2 refer to tumour cell lines.

whole with the immunofluorescence results (Fig. 2 and 3) and with the earlier finding that cells of MD cell lines are of thymic origin and that they carry tumour-specific antigens (Powell et al. 1974). No significant enhancement in uptake of anti-MDV serum was noted as a result of IdUrd treatment (Table 1). It is possible that antigens induced by IdUrd were intracellular, as are the EA and VCA antigens of IdUrd-activated Burkitt's lymphoma cell lines (Hampar et al. 1974), and were thus not detectable using unfixed cells. Induction of 'early antigens' in MSB-1 cells by treatment with IdUrd has recently been claimed by Nazerian (1975). The convalescent MD serum used here was MDV-specific since it reacted with MDV-infected cells (ratios 2:66 and 2:00) but failed to react with RSV-infected cells (ratio 0:85). Conversely, RSV antiserum which is used here as a control reacted only with RSV-infected cells (ratio 2:29).

Cells obtained from experiment 2 were also processed for autoradiography. The results obtained (Fig. 4) were consistent with those of 'bulk counting' and showed that cell surface virus-specific antigens were present in only a small proportion of MSB-1 cells and were negligible in HPRS-line 2. They showed further that both thymus and tumour-specific antigens were present at the surface of the majority of the cells of both cell lines as noted previously for HPRS-line 1 using immunofluorescence (Powell et al. 1974).
Table 2. Ig receptors in normal thymus cells and in lymphoblastoid cell lines

<table>
<thead>
<tr>
<th>Cells*</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
<th>Mean %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal thymus</td>
<td>55/213†</td>
<td>20/180</td>
<td>17/100</td>
<td>16</td>
</tr>
<tr>
<td>Line 2</td>
<td>0/200</td>
<td>0/200</td>
<td>0/400</td>
<td>0</td>
</tr>
<tr>
<td>MSB</td>
<td>0/200</td>
<td>0/200</td>
<td>0/100</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cells were treated with 1/20 dilution of anti-sheep erythrocyte serum (titre 1/1280 for 50% haemagglutination) washed and treated with sheep erythrocytes as described in Methods.
† Cells binding 3 or more erythrocytes

Total cells examined

Expression of normal T cell markers in cell lines

Studies using iodinated antibody

Expression of normal thymus cell markers was studied initially by estimating the uptake of iodinated anti-rabbit Ig by viable cells pre-treated with rabbit anti-thymus antibody. The results of two experiments shown in Table 1 suggested that anti-thymus serum reacted less strongly with MSB-1 and HPRS-line 2 cells than with normal thymus cells. Thus, in experiment 1, the ratio obtained in the case of normal thymus cells was 3.43 compared to 2.53 and 2.28 for the cell lines. Corresponding figures in experiment 2 were 2.88 for normal thymus cells and 1.37 and 1.87 for MSB-1 and HPRS-line 2 respectively. Autoradiography (Fig. 4) confirmed that anti-thymus serum reacted strongly with the majority of normal thymus cells but less so with MSB-1 and HPRS-line 2 cells.

Further differences in surface properties of normal thymus cells and cell lines were noted when cells were examined for the presence of Ig receptors. Table 2 shows that 16% of normal thymus cells obtained from bursectomized birds bound sheep erythrocytes after treatment with anti-sheep erythrocyte serum. The majority of the positive cells were lymphocytes as shown by Natt & Herrick’s stain (Natt & Herrick, 1952). Treatment of cells with normal rabbit serum followed by sheep erythrocytes or with sheep erythrocytes alone did not result in binding, suggesting that binding was attributable to the presence of Ig receptors on normal thymus cells. MSB-1 cells and HPRS-line 2 cells treated with anti-sheep erythrocyte serum invariably failed to attach sheep erythrocytes. Two explanations for the absence of Ig receptors in the lymphoblastoid cells are possible. (1) In vitro cultivation of lymphoma cells favoured the growth of a population of thymus cells which lack Ig receptors, (2) transformation has resulted in the loss of Ig receptors. We cannot distinguish between these possibilities because normal thymus cells are heterogeneous and have not so far been transformed in vitro. It is clear, however, that herpesvirus-induced Ig receptors were not present in MSB-1 cells and in HPRS-line 2 cells.

Characterization of external proteins associated with thymus-specific markers

Fig. 5 shows the results of analysis of external proteins of normal thymus cells and of tumour cells labelled by lactoperoxidase-catalysed iodination. Four main regions of radioactivity were noted when extracts of normal thymus cells were analysed in 8.5% gels (Fig. 5a). Approx. 50% of the activity in peak I was non-dialysable and was probably associated with lipids or lipoproteins because of their solubility in chloroform-methanol.
These materials which are of low mol. wt. (less than 14000), were not detected in immunoprecipitates obtained with anti-thymus serum and were not antigenic. Most of the activity present in extracts of cell lines was associated with materials which migrated to peak I. A small trypsin-sensitive polypeptide (peak II) having an apparent mol. wt. of 20000 was also resolved in extracts of normal thymus cells but its significance has not so far been determined.

Materials in the mol. wt. range 45000 to 67000 (peak III) and 90000 to 150000 (peak IV) were resolved in extracts of normal thymus cells and were not observed in MSB-1 cells or HPRS-line 2. These materials were trypsin-sensitive and consisted of multiple poly-
peptides. At least three and possibly four polypeptides were associated with thymus-specific antigenic determinants as shown by analysis of immunoprecipitates obtained with anti-thymus serum (Fig. 5b). Sixty-seven % of the total activity precipitated by anti-thymus serum (approx. 10 % of total TCA precipitable activity in the original sample) was associated with polypeptides in the mol. wt. range 45000 to 67000, indicating that these were the major molecular species carrying thymus-specific determinants that were exposed at the cell surface. Two additional polypeptides in the mol. wt. range 90000 to 150000 were also thymus-specific but were either relatively less exposed at the cell surface or had lower contents of tyrosine and histidine, judged by their comparatively lower
activity. When immunoprecipitates were analysed in 7.5% gels, (Fig. 6), the major exposed thymus-specific antigenic polypeptides were resolved into 2 distinct components of approximate mol. wt. 47,000 and 58,000, representing 20% and 50% of the total counts precipitated respectively. The 47,000 mol. wt. polypeptide was also resolved when immunoprecipitates obtained by reacting extracts of MSB-1 cells and HPRS-line 2 cells with anti-thymus serum were analysed (not shown) and probably contributes to the cross-reactions observed between normal thymus cells and the cell lines using anti-thymus serum. In addition, thymus-specific determinants might be associated with carbohydrate moieties exposed at the cell surface. However, the major thymus-specific polypeptide exposed at the surface of normal thymus cells (mol. wt. 50,000 to 58,000) were not accessible to iodination in the MSB-1 cell line and in HPRS-line 2 (not shown). These results thus provide a molecular basis for the lower reactivity of anti-thymus serum for lymphoblastoid cell lines compared to normal thymus cells as shown by the results of antibody uptake estimated by autoradiography (Fig. 4) and bulk counting (Table 1).

**DISCUSSION**

These experiments have shown that (1) the proportion of cells which express MDV antigens either spontaneously or after induction with IdUrd differs markedly between MSB-1 and HPRS-line 2 and does not exceed 10%. (2) The majority of cells of both cell lines express tumour-specific antigens which are serologically distinct from virus-specific antigens and (3) two or three polypeptides which carry thymus-specific antigenic determinants at the surface of normal thymus cells are modified in the cell lines and one of them probably also carries tumour-specific determinants.

The marked differences observed in spontaneous production of virus antigens and of infectious virus in the cell lines (Fig. 2 and 3) are reminiscent of the heterogeneity of virus expression in cells transformed by SV40 and Epstein-Barr viruses. By analogy to EBV cell lines (Adams, Strander & Cantell, 1975), MSB-1 cells might be classified as producer cells and HPRS-line 2 as non-producer cells. However, as there is evidence of a decline in the capacity to rescue infectious virus upon continuous growth, the distinction between MSB-1 and HPRS-line 2 might reflect the difference in ‘age’ of these cultures rather than intrinsic differences. We attribute the decline in proportion of infective centres during continuous subculture to selection of non-producer cells. A 10-fold difference in the proportion of cells synthesizing virus antigens was noted between HPRS-line 2 and MSB-1 cells, but in contrast to the proportion of cells scoring as infective centres, the proportion of cells synthesizing virus antigens fluctuated but did not decline significantly during the course of these experiments. It is possible that continuous subculture favoured the growth of those MSB-1 cells which express virus functions compatible with cell division and that eventually these might also disappear as is evident in HPRS-line 2.

The factors that control virus expression in cell lines are unknown. The fact that treatment with IdUrd activated virus expression proves that virus genome is present in a repressed form in many more cells than are normally detected. Inhibition of cell division as such using 1 mM-theophylline and cyclic AMP (unpublished results) did not result in activation. Conversely, treatment of MDV-infected CEF with IdUrd did not enhance the infective centres or the cells synthesizing antigens (unpublished results). It is thus likely that, as has been found with EBV and SV40, IdUrd specifically rescues repressed MDV and allows transcription and translation of the virus genome to take place. There was evidence that additional host-determined permissive factors also contribute to the control of virus
expression. This was shown by the fact that IdUrd treatment did not affect synthesis of virus antigens in HPRS-line 2 despite the observed enhancement of infective centres when treated cells were plated on the permissive CKC. Furthermore, the plaques formed by activated HPRS-line 2 cells were very slow to develop compared to those formed by MSB-1 cells, suggesting that additional factors determine virus expression. The nature of these permissive factors is unknown, but the results of a study of the precipitating antigens that are expressed in MSB-1 cells discussed below are consistent with the hypothesis that failure to synthesize some virus antigens is attributable to a deficiency in host components as has been found for EBV (Nyormoi et al. 1973) and for SV40 (Watkins, 1974).

Direct evidence for the presence of virus-specific antigens in extracts of MSB-1 cells was obtained by immunodiffusion (Fig. 1). At least two precipitins previously described as B and C antigens (Ross et al. 1975) were detected but the ‘A’ antigen, a major glycoprotein antigen (Ross et al. 1973) was not detected. The fact that virus isolated from MSB-1 and from HPRS-line 2 had retained the ability to induce A antigen when grown in permissive CKC provides strong evidence for host-determined factors which control expression of this antigen.

It is of interest that although extracts of HPRS-line 2 cells did not form precipitins with convalescent serum after 20 months in culture (Fig. 1), the virus isolated from HPRS-line 2, a derivative of HPRS-16, was still pathogenic and had retained the ability to induce A, B and C antigens when grown in CKC. This suggests that attenuation of HPRS-16 by serial passage in CKC and the concurrent loss of the A antigen reported earlier (Churchill et al. 1969) is not an inevitable consequence of long term cultivation in vitro but depends rather on host cell factors and perhaps on the nature of the association between virus genome and host genome.

The lack of identity between virus antigens and tumour-specific antigens reported earlier (Powell et al. 1974; Witter et al. 1975) was re-examined here by estimating the uptake of iodinated anti-Ig in an indirect serological test. The results of both ‘bulk counting’ (Table 1) and of autoradiography (Fig. 4) confirmed the earlier results that virus and tumour-specific antigens are serologically distinct. This was shown by the fact that anti-tumour serum reacted with tumour cell lines but not with MDV-infected CEF and conversely by the fact that convalescent MD serum used here as a source of anti-MDV antibodies reacted with the majority of MDV-infected cells but only with a small proportion of the cells of the producer cell line MSB-1 and only marginally with HPRS-line 2.

Marked differences in surface properties of cell lines and of normal thymus cells were noted. Prominent among these was the finding that the major thymus-specific polypeptide (mol. wt. 58,000) (Fig. 6b) was not accessible to lactoperoxidase-catalysed iodination in the cell lines despite clear evidence of the expression of thymus-specific antigens as shown by other tests (Fig. 4, Table 1). The failure to iodinate external proteins of viable growing tumour cells is not surprising and is consistent with the recent reports that a high mol. wt. glycoprotein exposed at the surface of normal cells is no longer available to iodination in cells transformed either by viruses or by carcinogens (Hynes, 1973; Pearlstein et al. 1976). This effect has been attributed by some to rapid turnover and to secretion into culture medium (Keski-Oja, Vaheri & Ruoslahti, 1976) and to proteolysis by others (Roblin, Chou & Black, 1975). The results of uptake of anti-thymus antibody by viable cells of MSB-1 and HPRS-line 2 (Table 1) provide good evidence for the presence of some thymus-specific antigens in cell lines. We conclude therefore, that thymus-specific antigens have been sterically or chemically modified in cell lines and that they are not exposed to the
same extent as in normal cells. Our results are consistent with those of Matsuda, Ikuta & Kato (1976), who have recently reported that several cell lines derived from Marek's disease lymphomas reacted less strongly with anti-thymus antibody in immunofluorescence and cytotoxicity tests compared to normal thymus cells. Further differences in surface properties between normal cells and cell lines were demonstrated when cells were examined for the presence of Ig receptors.

A search for Ig receptors in cell lines as evidence of herpesvirus expression as found in cells transformed by herpes simplex (Westmoreland, Watkins & Rapp, 1974) was negative. These results suggest that expression of Ig receptors is not an invariable consequence of transformation by all herpesviruses. Paradoxically, 16% of normal thymus cells reacted with rabbit Ig. At this stage, it is not known whether the absence of Ig receptors in cell lines is attributable to their deletion as a result of transformation or to selection of cells lacking Ig receptors during continuous growth in vitro, because normal thymus cells are heterogeneous, and transformation of pure lymphocytes by MDV in vitro has not been achieved. Differences in the response of tumor cell lines and normal thymus cells to PHA stimulation noted previously (Powell et al. 1975) also provide evidence for surface differences between normal and tumor cells. However, it is not possible to attribute this directly to transformation for reasons adduced above for the absence of Ig receptors in cell lines.

We wish to thank Mr Brian Whitby for excellent technical assistance; Dr R. M. Dougherty and Dr P. M. Biggs for many valuable suggestions. It is also a pleasure to thank Mr R. Sampson for the photography and Miss Helen Brandon for typing the manuscript.

REFERENCES


Antigen expression in MD cell lines


(Received 22 September 1976)