GLYCOPROTEINS OF THE AKR LEUKAEMIA CELL
SURFACE AND THEIR RELEVANCE TO LEUKAEMIA-
specific surface antigens

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SUMMARY
An attempt has been made to prepare antibodies against leukaemia-specific surface antigens by
immunizing (C57Bl/6 × C3H/HeJ)F1 mice with formaldehyde-stabilized AKR leukaemic cells.
The presence of antibodies was examined by indirect immunofluorescence microscopy (IFM) and
the indirect antiglobulin rosetting reaction (IARR). Galactose oxidase treatment destroyed the
ability of leukaemic cells to react with antibodies prepared in the hybrid mice, an effect that was
reversed by treating the enzyme-modified cells with borohydride. Analysis by immunoprecipitation
and polyacrylamide gel electrophoresis of leukaemic cells, labelled by the galactose oxidase/[3H]-
NaBH₄ technique, indicated that a group of glycoproteins of apparent molecular weight >70000
was involved. Antibodies could be raised in AKR mice to the same group of glycoproteins by
immunization with irradiated leukaemic cells or irradiated neuraminidase-treated leukaemic cells.
The level of antibody raised in AKR mice had no effect on the growth of leukaemic lymphoblasts
introduced subcutaneously into the host.

Antibodies prepared in hybrid mice against leukaemic cells also were absorbed by lymphoid cells
of pre-leukaemic 6-month-old AKR mice, indicating that contrary to previous claims in the
literature antigens detected by such antisera are not related to malignancy. Hybrid mouse serum
cross-reacted with antigens from purified RNA virus isolated from Abelson lymphoma, as demon-
strated by the immunoelectrophoretic blotting technique. The pattern of reactivity was not appreci-
ably altered following the absorption of antibodies directed against leukaemic cells. It is concluded
that the glycoproteins detected by us may not be viral antigens but normal high molecular weight
lymphoid glycoproteins with altered glycosylation patterns that are induced when the viral genomes
are expressed.

INTRODUCTION
The tumour cell surface is the focus of much contemporary research, because it is
considered that alterations in the plasma membrane may not only account for changes in
behavioural characteristics of tumour cells but also provide targets for immune
recognition and destruction of these cells (Hogg, 1979). In the case of mouse anti-
leukaemia sera, the specificities appear to be directed towards virion structural
proteins; little success has been achieved in producing activity against non-virion, but
tumour-specific, antigens. Okazaki, Aoki & Dekozlowski (1976), examining antisera
against five newly established AKR spontaneous leukaemias, have put forward
evidence that they consider demonstrates the presence of leukaemia-specific antigens,
acquired as a result of malignant transformation at the surface of the leukaemic cell.
The biochemical nature of these antigens was not reported. We have prepared an
antisera against leukaemic lymphoblasts of AKR mice following the methods
described by Okazaki et al. (1976) and have demonstrated, by immunofluorescence microscopy and additionally by an indirect antibody rosetting reaction, the presence of antibodies interacting with components at the surface of the leukaemic cells. It is of particular interest to find that the immunofluorescence assay and the indirect antibody rosetting reaction are dependent on terminal non-reducing galactosyl residues that may be reversibly modified by treatment with galactose oxidase and borohydride. Exposure of additional galactosyl residues to neuraminidase enhances the rosetting reaction. Analysis by immunoprecipitation and polyacrylamide gel electrophoresis (PAGE) indicates that our antiserum is reacting with glycoproteins of apparent molecular weights >70000. The inoculation of AKR mice with irradiated leukaemic cells, with or without neuraminidase treatment, results in the production of antibodies reacting with the same group of glycoproteins. The level of antibody raised in these experimental animals had no effect on the growth of leukaemic lymphoblasts introduced subcutaneously into the host.

We found that the antibodies prepared against leukaemic cells by the methods of Okazaki et al. (1976) are completely absorbed by lymphoid cells of 6-month-old preleukaemic AKR mice. Additionally, we observed that the pattern of viral antigens cross-reacting with hybrid mouse serum is not appreciably altered by the absorption of antibodies directed against leukaemic cells. In the light of these results we suggest that claims (Okazaki et al. 1976) to have demonstrated leukaemia-specific antigens acquired by malignant transformation must be treated with caution. It is concluded that the glycoproteins detected by us may not be viral antigens but normal high molecular weight lymphoid glycoproteins (Gahmberg, Häyry & Andersson, 1976) with altered glycosylation patterns that are induced when the viral genomes are expressed.

MATERIALS AND METHODS

Mice

AKR mice were routinely purchased from OLAC Ltd, Bicester, Oxon, England. C57B1/6 and C3H/He mice were obtained from National Institute for Medical Research, Mill Hill, London, England and (C57B1/6 × C3H/He)F1 hybrids were bred at the Central Animal Services Department, University of Cambridge, England.

Leukaemia

A spontaneous AKR leukaemia obtained from Dr W. Jacobson, Strangeways Research Laboratory, Cambridge, was transmitted in AKR mice as described in detail by Cook & Jacobson (1968); cells used for immunization were obtained from ninth to fiftieth generations from the initial transplantation. Tumours were removed 12 days after subcutaneous implantation and a cell suspension was prepared by mechanical dissociation (Rupar & Cook, 1982). This population of cells was freed from erythrocytes and dead cells by centrifuging the cell suspension on an Isopaque/Ficoll barrier and recovering the cells collecting at the interphase using the method described by Davidson & Parish (1975). Routinely, $2 \times 10^7$ cells with >90% viability, as determined by Trypan Blue exclusion, were obtained from each 0·5 g (wet weight) tumour.

Some experiments, where stated, were performed with leukaemic cells established in tissue culture in Earle's Minimal Essential Medium supplemented with 5% foetal bovine serum, gentamycin (50 μg/ml) and Fungizone (1·5 μg/ml).
Antisera

Four groups of ten 1- to 3-month-old (C57B1/6 × C3 H/He)F1 hybrid mice were immunized with formaldehyde-stabilized (Kudo, Aoki & Morrison, 1974) AKR leukaemic cells, serially increased in number from 10⁶ to 10⁸ cells per inoculation, as described in detail previously (Okazaki et al. 1976). After the fourth immunization, pooled serum was taken by tail-bleeding. A further four inoculations with 10⁸ formaldehyde-stabilized cells were performed and sera were obtained after each inoculation. As described by Okazaki et al. (1976), all undiluted antisera and preimmune sera were absorbed twice in vitro with non-leukaemic thymuses, lymph nodes and spleens of 1 to 2-month-old AKR mice, to remove antibodies against alloantigens, using the procedure of Old, Boyse & Stockert (1965). Some samples of undiluted antisera were also subjected to further absorption in vitro with thymuses, lymph nodes and spleens of 6-month-old pre-leukaemic AKR mice. Antisera were absorbed also with Balb/c embryonic tissues (Okazaki et al. 1976) as a control for the presence of antibody to foetal antigen. In addition, multiple absorptions were performed with lymphoid cells of 2-month-old AKR mice as well as leukaemic cells.

The anti H-2/K serum used as a control was raised in CBA mice against AKR lymphocytes and was kindly supplied by Dr I. McConnell, MRC Centre, Cambridge. This antiserum was routinely used at 1/500 final dilution.

Immunofluorescence microscopy (IFM)

Indirect IFM was carried out by the method of Möller (1961) as described by Okazaki et al. (1976), with the exception that bound immunoglobulin (lg) was detected with fluorescein-conjugated rabbit anti-mouse IgG (molar ratio F/P = 4:4; Miles Laboratories, Slough, England). The preparations were examined in a Leitz Ortholux fluorescence microscope.

Indirect antiglobulin rosetting reaction (IARR)

Surface bound antibody was detected using the procedure described by Bright et al. (1977). The indicator cells used in this reaction were trypsin-treated sheep red blood cells coupled to an IgG fraction of sheep anti-mouse IgG antiserum and generously supplied by Professor R. R. A. Coombs, Department of Pathology, University of Cambridge.

For each serum tested 200 lymphoid cells were counted and the percentage of rosetted cells binding a minimum of four indicator erythrocytes was recorded. All determinations were performed in duplicate with coded samples. Control experiments using sheep erythrocytes coupled to non-immune sheep IgG were performed in every case.

Direct antiglobulin rosetting reaction (DARR)

The direct antiglobulin rosetting reaction was performed on leukaemic cells using the method described by Coombs et al. (1977). Care was taken to examine washed suspensions of leukaemic cells immediately following their preparation, in order to avoid the agglutination of the anti-globulin-coupled indicator sheep erythrocytes with immunoglobulin released from the murine cells. At least 200 cells were examined for each determination and control experiments with sheep erythrocytes coupled to non-immune sheep IgG were performed in every case.

Presence of Fc receptors

In order to test for the presence of Fc receptors on the leukaemic cell surface, the opsonic adherence rosetting reaction of Hallberg, Gurner & Coombs (1973), in which ox erythrocytes sensitized with the IgG fraction of rabbit anti-ox-erythrocyte serum were used as indicator cells, was employed.

Labelling of cell surfaces with biotinylated Ricinus communis agglutinin

Glutaraldehyde-fixed leukaemic cells (2 × 10⁶ cells/ml) were treated with neuraminidase (Cook & Jacobson, 1968) and the exposure of galactosyl residues was monitored by treating the cells with biotinylated R. communis agglutinin (125 μg RCA₁₂₀/2 × 10⁶ cells; Vector Labs, Calif, U.S.A.)
for 5 min on ice. Lectin-mediated biotinylation was visualized by treating the cells with ferritin-labelled avidin (Vector Labs) as described by Skutelsky, Danon, Wilchek & Bayer (1977). Analysis of labelling density was performed on electron micrographs of perpendicularly sectioned membranes as detailed by Skutelsky et al. (1977). Control experiments were performed with non-enzyme-treated cells as well as samples in which the lectin was added in the presence of 50 mM-lactose.

Radiolabelling of cell surface proteins

Exposed galactose residues at the leukaemic cell surface were radiolabelled by modification of the methods of Gahmberg et al. (1976), Andersson, Gahmberg, Nilsson & Wigzell (1977) and Andersson et al. (1979).

Leukaemic cells (2 × 10^7 cells/ml) were incubated at 37°C for 30 min in RPMI 1640 medium together with galactose oxidase (5 units/ml protease, neuraminidase-free and glucose oxidase-free; Kabi Diagnostica, Sweden) with or without neuraminidase (12-5 units/ml protease and lecinthinase-free; Behringwerke AG, W. Germany); units of neuraminidase activity are as defined by Schramm & Mohr (1959). In control experiments the enzymes were omitted. Following incubation the cells were washed twice in 30 vol. of RPMI 1640 at 500 g for 5 min at 4°C. The washed cells were resuspended in RPMI 1640 (0.5 ml) and incubated with 0.5 mCi NaB₃¹H₄ (424 mCi/mmol; Amersham International, Bucks, England) on ice for 10 min. Then the cells were washed three times in RPMI 1640 and once in Dulbecco's phosphate-buffered saline (PBS), suspended in PBS (2.0 ml) and a sample was counted for radioactivity in a toluene/Triton X-100 (2:1, v/v)-based scintillation fluid (Rupar & Cook, 1982). The cell viability, as assessed by Trypan Blue dye exclusion, remained >90% throughout the above procedure.

Immunoprecipitation and PAGE

The ³H-labelled leukaemic cells (2 × 10⁷) resuspended in 150 μl PBS were immunoprecipitated with an equal volume of antiserum. After incubation for 1 h at room temperature the cells were washed twice in 15 vol. PBS (500 g, 5 min) and solubilized with 0.1% NP40 in 50 mM-Tris (pH 7-4) containing 2 mM-phenylmethylsulphonyl fluoride (150 μl). After incubation on ice for 30 min following centrifugation for 1 h at 100 000 g, the supernatant fluid was removed and 60 μl protein A-Sepharose CL-4B (Pharmacia) added. The antibody–antigen complexes that bound to the protein A-Sepharose during the 30 min incubation at room temperature were washed three times in 10 vol. PBS. Antigen was removed from the immobilized protein A by boiling in 100 μl 2% sodium dodecyl sulphate (SDS) in 100 mM-Tris (pH 7-2) for 5 min followed by centrifugation at 9950 g. Samples of the supernatant fluid were monitored for radioactivity and binding to R. communis lectin (RCA120) − agarose (1-1 mg protein/ml resin; Miles Laboratories). Binding to immobilized lectin was performed on samples diluted 100-fold with 50 mM-Tris buffer (pH 7-2) and was controlled by measuring uptake of radiolabel in the presence and absence of 100 mM-lactose. Quantities of the supernatant fluid were treated with mercaptoethanol (2% final concentration) at 100°C for 5 min and examined in 7.5% PAGE (Fairbanks, Steck & Wallach, 1971). The cylindrical gels were cut into 2-mm slices, solubilized in NCS tissue solubilizer (0.5 ml; Amersham-Searle) overnight at 4°C and counted in toluene/ethoxyethanol (7:3, v/v)-based scintillation fluid (Rupar & Cook, 1982).

Examination of sera for anti-viral activity

Samples of sera from hybrid mice were kindly examined for antiviral activity by Dr G. L. E. Koch and Mr R. A. Mortara, MRC Laboratory of Molecular Biology, Cambridge. Purified RNA virus isolated from Abelson lymphoma (Koch, G. L. E. & Mortara, R. A., unpublished) was fractionated by SDS/PAGE on a 10% /14% step slab gel and electrophoretic blots prepared on nitrocellulose sheets (Towbin, Staehelin & Gordon, 1979). The blots were incubated for 6 h with 1/20 dilutions of antiserum. Following extensive washing the blots were treated with affinity-purified ¹²⁵I-labelled rabbit anti-mouse IgG (1.5 × 10⁵ c.p.m./ml) and after further extensive washing the blots were dried and subjected to autoradiography. A control blot was incubated with rat anti-pI5(E) and developed with ¹²⁵I-labelled sheep anti-rat IgG (1.5 × 10⁴ c.p.m./ml).
Inoculation of AKR mice with irradiated leukaemic cells

A group of 1- to 2-month-old AKR mice were inoculated intraperitoneally with \(10^6\) irradiated leukaemic cells (10,000 rad at 60 rads/s) in Earle's balanced salt solution (EBSS 0.2 ml). A further three inoculations of \(10^7\) irradiated cells were given at intervals of 2 weeks. Another group of mice was subjected to the same inoculation regimen but with irradiated leukaemic cells that had been subjected to neuraminidase (Cook & Jacobson, 1968). A control group was injected with EBSS only. One week after the last inoculation all the animals were given a subcutaneous implant of leukaemic cells (Cook & Jacobson, 1968) and mean tumour diameters were measured (Lachmann & Sikora, 1978) from the 12th day onwards.

RESULTS

Examination of antiserum to leukaemic cells

Serum taken from F1 hybrid mice prior to administration of leukaemic cells gave a negative reaction in the indirect IFM assay, while 10.3(±1.4)% (38) (mean ± s.e.m.; number in parenthesis refers to separate determinations) of leukaemic cells showed a positive IARR. When the IARR was repeated with cultured leukaemic cells, only 2% of the target cell population formed rosettes. After the fourth inoculation of leukaemic cells (Okazaki et al. 1976), the antiserum reacted with 47(±3.0)% (3) cells as measured by indirect IFM and lacked rosetting above the pre-immune level (Fig. 1A). After six inoculations the percentage of cells exhibiting immunofluorescence had risen to 65(±4.7)% (6) and the IARR had increased to 20(±1.2)% (3). Following the seventh and eighth inoculations, the assay values had risen to 70(±1.6)% (7) and 78(±1.3)% (4) for indirect IFM and 37.8(±2.7)% (12) and 48.8(±2.2)% (18) for the IARR, respectively. Fig. 1B, C shows typical immunofluorescence and IARR, respectively. All further experiments were performed using serum taken after the seventh or eighth inoculation. In all experiments viable leukaemic cells exhibited a positive reaction by indirect IFM 92(±4.6)% (19) and the IARR 90(±2.7)% (49) with anti-H-2/K serum. A direct IFM assay between leukaemic cells and FITC-labelled anti-mouse IgG was consistently negative.

When antiserum that had been routinely absorbed twice with lymphoid cells from 1- to 2-month-old AKR mice was subjected to a further four absorptions with these cells and tested against viable leukaemic cells there was no change in the level of immunofluorescence. However, when antiserum was absorbed twice more with lymphoid cells from 6-month-old, preleukaemic AKR mice the immunofluorescence reaction dropped from 78% to 4%. A similar result was obtained with anti-serum absorbed twice with viable leukaemic cells. Two absorptions of antiserum from immunized hybrid mice with embryonic tissue did not lower the level of antibody as detected by the IARR.

Endogenous surface immunoglobulin and Fc receptors on leukaemic cells

No endogenous surface immunoglobulin was detected by indirect IFM on untreated leukaemic cells. However, 11(±0.6)% (38) of leukaemic cells gave a positive DARR. Following the removal of these rosettes through a Ficoll–Isopaque
Pre-immune serum 4th 6th 7th 8th Serum after inoculations Anti-H2/K serum

Figs 1A–C
barrier, a value of 2.5 (± 0.9) % (3) was obtained when the DARR was repeated on the non-sedimenting cells. A similar level (2%) was obtained when a DARR was carried out on leukaemic cells established in tissue culture.

The opsonic rosetting reaction for the detection of Fc receptors demonstrated that 13 (± 2.5) % (11) of the cells within the leukaemic cell suspensions formed rosettes. Following the removal of cells forming rosettes in the DARR through a Ficoll/Isopaque barrier, the percentage of non-sedimenting cells showing the presence of Fc receptors was reduced to 3.5%.

Availability of galactosyl residues

An estimate of the number of terminal β-galactosyl residues available at the surface of the leukaemic cell was made using biotinylated RCA120 and ferritin-labelled avidin. The density of ferritin particles on leukaemic cells treated with the biotinylated lectin alone was 4.3 (± 0.5) particles/μm (10). Following the enzymic removal of surface sialic acid the particle count rose to 20.7 (± 1.4) particles/μm (10). Doubling the concentration of biotinylated lectin to 250 μg RCA120/2×10⁶ cells had

Fig. 1. A. Production of antibody in F₁ hybrid mice against AKR leukaemic cells as assessed by the percentage of leukaemic cells showing a positive reaction in the indirect IFM (open histogram) and IARR (cross-hatched) assays. The procedures used are those described in Materials and Methods. Serum from the hybrid mice was assayed at a 1/2 dilution after absorption with lymphoid cells from 2-month-old AKR mice; anti-H-2/K serum was diluted 1/500. Mean ± S.E.M. values are quoted. B. A typical immunofluorescence 'ring' reaction exhibited by viable leukaemic cells treated with absorbed hybrid mouse serum (after 7th inoculation). ×1000. C. A typical IARR between leukaemic cells treated with absorbed hybrid serum (after 8th inoculation) and sheep erythrocytes coupled to sheep anti-mouse IgG. Leukaemic cells are stained with Toluidine Blue. ×500.

Fig. 2. Perpendicular view of leukaemic cells treated: A, with biotinyl RCA120 and ferritin-labelled avidin; B, with biotinyl RCA120 and ferritin-labelled avidin after neuraminidase treatment; and C, with biotinyl-labelled RCA120 applied in the presence of 50 mM-lactose followed by ferritin-labelled avidin. ×96 000.
no effect on the particle counts. When the lectin was applied in the presence of 50 mM-lactose, the sections showed no adherent particles (Fig. 2).

Exposure of additional galactosyl and N-acetylgalactosaminyl residues by treatment with neuraminidase was also demonstrated by following the incorporation of $^3$H from $[^3]$HNaBH$_4$ after treatment with galactose oxidase. The level of radio-label incorporated after galactose oxidase treatment for 30 min was $2.9(\pm0.9)\times10^5$ d.p.m./$10^7$ cells (3) as compared to $2.0(\pm0.6)\times10^5$ d.p.m./$10^7$ cells (5) without enzyme treatment. The incorporation of $^3$H was greatly enhanced to $1.1(\pm0.2)\times10^6$ d.p.m./$10^7$ cells (5) by the addition of neuraminidase to the reaction mixture, and 68% of this radio-label is solubilized by 0.1% NP40. A further 5% of the radio-label can be solubilized with 1% NP40.

**Effect of enzymes on antibody binding to leukaemic cells**

The binding of antibody to leukaemic cells treated at 37°C with neuraminidase (12.5 units/$2\times10^7$ cells) for 30 min was increased by 10%, as assessed by indirect IFM. Galactose oxidase treatment (5 units/$2\times10^7$ cells) for 30 min reduced the control value of 77% cells showing a positive indirect IFM reaction to 8%. This effect was reversed to 69% by subsequent treatment with NaBH$_4$ ($2\times10^7$ cells treated with 1.3 mM-NaBH$_4$ in EBSS (1 ml) for 10 min at room temperature). Neither enzyme affected the binding of anti-H-2/K serum to the leukaemic cells.

The effect of treating the leukaemic cells with neuraminidase, galactose oxidase and combinations of these enzymes on the IARR is illustrated in Fig. 3. None of the experiments shown in Fig. 3A involved the use of radio-labelled cells, whilst in Fig. 3B all the cells were subjected to a period of reduction with $[^3]$HNaBH$_4$.

In the case of leukaemic cells treated with galactose oxidase, there is a rapid drop in the IARR from 45.2 (±0.7)% (3) to 11.2 (±2.7)% ($\pm$ range of 2 determinations) over the first half hour and by 1 h a drop to 10.2 (±0.6)% (4), which is significantly ($t$-test, $P<0.001$) different from the control value (Fig. 3A). A similar result was achieved when the cells were treated with neuraminidase for various periods up to 1 h followed by 1 h incubation with galactose oxidase (5 units). A less dramatic reduction was achieved when the initial treatment involved both galactose oxidase (5 units) and neuraminidase (12.5 units), followed by galactose oxidase (5 units).

None of these results has been corrected for the contribution made by cells bearing

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Fig. 3. The effect of treatment of leukaemic cells with neuraminidase and galactose oxidase on the binding of hybrid mouse antisera, as monitored by the IARR. a. Leukaemic cells treated with buffer (○); galactose oxidase (●); or neuraminidase for various periods then 1 h with galactose oxidase (△); neuraminidase and galactose oxidase for various periods then followed by 1 h with galactose oxidase at 37°C (□). The conditions of enzyme treatment are described in the Materials and Methods section on radio-labelling the cell surface proteins. b. Leukaemic cells treated with buffer (○); neuraminidase (▼); neuraminidase and galactose oxidase (▲). At each time-point treatment with $[^3]$H-NaBH$_4$ was done for 10 min at 0°C. Error bars indicate mean value ± s.r.m., except for the 30 min incubation with galactose oxidase, and all the cells treated with neuraminidase (alone or simultaneously with galactose oxidase) followed by galactose oxidase for 1 h, where mean value ± range is shown.
AKR leukaemia cell glycoproteins

Figs 3A and B
surface immunoglobulin. The DARR was carried out for each time-point and varied between 6·1 % and 14·4 %. Galactose oxidase treatment alone had no effect on the percentage of cells forming rosettes in the DARR. Correcting the values shown in Fig. 3A for the contribution made by cells bearing surface immunoglobulin, after 30 min treatment with galactose oxidase no cells formed rosettes with exogenous antibody.

Treating leukaemic cells for up to 1 h with $[^3H]$NaBH$_4$ did not significantly alter the IARR (40 (± 1·4) % (5), after 1 h treatment with $[^3H]$NaBH$_4$; 44·1 (± 1·7) % (3) for unlabelled cells after 1 h). Treatment with neuraminidase followed by $[^3H]$-NaBH$_4$ increased the percentage of cells exhibiting rosette formation (Fig. 3b). Of particular interest, however, is the finding that when cells are treated concomitantly with neuraminidase and galactose oxidase, and subsequently reduced with $[^3H]$-NaBH$_4$, the IARR is significantly increased, as compared to treatment of cells for 1 h with buffer and then with $[^3H]$NaBH$_4$ ($P < 0·001$). Under these conditions a maximum number of terminal galactosyl residues is available, as measured by the incorporation of radio-label (see 'Availability of Galactosyl Residues', above).

Characterization of labelled components interacting with antisera

The results of immunoprecipitation and PAGE of cells labelled with $[^3H]$NaBH$_4$ after digestion by neuraminidase and galactose oxidase are shown in Fig. 4. Material interacting with antiserum from hybrid mice inoculated with leukaemic cells is shown in Fig. 4A. The majority of the immunoprecipitated radio-label is associated with components of apparent molecular weight >70 000, with a prominent peak of 97 000. Of the labelled material subjected to PAGE, 82 % was adsorbed to RCA$_{120}$-agarose; in the presence of the competitive sugar, lactose, this binding is reduced to 15 %. When pre-immune serum was used no labelled components were detected by PAGE (Fig. 4A). In the case of antiserum obtained from AKR mice inoculated with irradiated leukaemic cells, either untreated or treated with neuraminidase, the sera reacted with a similar group of glycoproteins, as shown in Fig. 4B. However, in the case of the serum obtained from animals inoculated with neuraminidase-treated cells, approximately three times more radio-labelled material was recovered than with an equal quantity of serum obtained from animals inoculated with irradiated leukaemic cells only. Leukaemic cells labelled in the absence of neuraminidase and galactose oxidase possessed no material that reacted with immobilized protein A (lower trace in Fig. 4A). The majority of the immunoprecipitated radio-label is associated with components of apparent molecular weight >70 000, with a prominent peak of 97 000. Of the labelled material subjected to PAGE, 82 % was adsorbed to RCA$_{120}$-agarose; in the presence of the competitive sugar, lactose, this binding is reduced to 15 %. When pre-immune serum was used no labelled components were detected by PAGE (Fig. 4A). In the case of antiserum obtained from AKR mice inoculated with irradiated leukaemic cells, either untreated or treated with neuraminidase, the sera reacted with a similar group of glycoproteins, as shown in Fig. 4B. However, in the case of the serum obtained from animals inoculated with neuraminidase-treated cells, approximately three times more radio-labelled material was recovered than with an equal quantity of serum obtained from animals inoculated with irradiated leukaemic cells only. Leukaemic cells labelled in the absence of neuraminidase and galactose oxidase possessed no material that reacted with immobilized protein A (lower trace
AKR leukaemia cell glycoproteins

A. Sera from hybrid mice

- 97K
- 116K

B. Sera from AKR mice

- 99K
- 128K
- 105K
- 80K
- 75K

C. Anti-H2/K serum

- 47K
- 35K

Figs 4A–C
Fig. 5. An electrophoretic blot of proteins of purified virus isolated from Abelson lymphoma. The proteins were fractionated by SDS/PAGE using a 2-step 10% / 14% acrylamide slab gel and subsequently transferred to nitrocellulose paper by electrophoretic blotting; track a, blot reacted with unabsorbed hybrid mouse antiserum (after 6th inoculation); b, blot reacted with same hybrid mouse antiserum but absorbed twice with lymphoid cells from 2-month-old AKR mice; c, blot reacted with hybrid mouse antiserum absorbed with lymphoid cells from 2-month-old AKR mice followed by two absorptions with leukaemic cells. The immune complexes were detected by treatment with 125I-labelled affinity-purified rabbit anti-mouse IgG, as described in Materials and Methods. A blot (d) was treated with rat anti-p15(E) serum that is known to react with p15(E), p30 and p65\textsuperscript{AG}. Proteins reacting with this anti-serum were detected with 125I-labelled sheep anti-rat IgG. The gel from which the blots were prepared was stained with Coomassie Brilliant Blue and is shown in track e.
Fig. 4B). As a positive control, labelled cells were treated with anti-H-2/K serum: a major peak of 47,000 and a peak of 35,000 were demonstrated (Fig. 4c).

Viral components interacting with antisera

Fig. 5 shows an indirect autoradiograph of viral antigens reacting with serum from hybrid mice subjected to eight inoculations with leukaemic cells. In track a, unabsorbed serum is seen to cross-react with a number of viral proteins, including those of molecular weights 100,000, 65,000, 30,000 and 15,000; the first two components are almost certainly products of the gag gene, with the 65,000 band being the polyprotein precursor (p65<sup>gag</sup>) of the major structural proteins of the virus. Other components have been identified as the viral core polypeptide p30 and the envelope protein p15(E). Weakly immunoreactive material between the 100,000 and 65,000 bands is likely to be the glycosylated envelope protein gp70, which stains poorly with Coomassie Brilliant Blue (see track e). The pattern in track a is substantially unaltered by two absorptions with normal lymphoid cells from 2-month-old AKR mice (see track b). A further two absorptions with leukaemic cells, with the possible exception of the low molecular weight envelope components, does not appreciably alter this pattern; the serum continues to show a marked reaction against p30 and reacts with the 65,000 and 100,000 gag components. The rat anti-p15(E) is known to react with p30 and p65 (G. L. E. Koch, personal communication) and a blot was treated with this antibody (track d) to aid identification.

Immunization of AKR mice with irradiated leukaemic cells

When AKR mice, immunized with irradiated tumour cells with or without neuraminidase treatment, were injected subcutaneously with viable leukaemic cells, no difference was found in the progression of the tumour between the treated and untreated animals, as assessed by measurement of tumour diameter (Table 1). All the animals had died or were at the point of death by the 16th day. Serum from both groups of immunized mice exhibited a positive reaction with leukaemic cells in direct IFM and in the IARR.

DISCUSSION

Okazaki et al. (1976) claim to have raised antibodies to leukaemia-specific surface antigens by immunizing hybrid mice with cells from spontaneous leukaemias of AKR mice. We have repeated their procedures in an attempt to define the biochemical nature of such antigens. When testing the antisera from hybrid mice against leukaemic cells, we found that only 47% of the cells showed a positive reaction by indirect IFM, as compared to >70% demonstrated by Okazaki et al. In order to obtain sera that would produce comparable levels of immunofluorescent cells, we found it necessary to give the hybrid mice four more inoculations of leukaemic cells. As well as using indirect IFM, we have examined our antisera by means of the IARR. Both of these assays would be complicated by the presence of endogenous immunoglobulin at the
Table 1. Mean tumour diameters produced following the subcutaneous implantation of viable leukaemic cells in to AKR mice previously treated with irradiated leukaemic cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean tumour diameter (cm)* on day:</th>
<th>Indirect IFM†</th>
<th>Target cells</th>
<th>IARR†</th>
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<tr>
<td></td>
<td>12</td>
<td>13</td>
<td>14</td>
<td>15</td>
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<tr>
<td>Irradiated leukaemic cells in RPMI 1640</td>
<td>1.4 ± 0.07</td>
<td>1.6 ± 0.07</td>
<td>1.9 ± 0.09</td>
<td>2.0 ± 0.08</td>
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<td>(14)</td>
<td>(14)</td>
<td>(11)</td>
<td>(4)</td>
</tr>
<tr>
<td>Neuraminidase-treated irradiated</td>
<td>1.5 ± 0.07</td>
<td>2.0 ± 0.04</td>
<td>2.0 ± 0.05</td>
<td>2.1 ± 0.11</td>
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<tr>
<td>leukaemic cells in RPMI 1640</td>
<td>(27)</td>
<td>(25)</td>
<td>(19)</td>
<td>(8)</td>
</tr>
<tr>
<td>RPMI 1640‡</td>
<td>1.4 ± 0.70</td>
<td>1.6 ± 0.11</td>
<td>2.0 ± 0.14</td>
<td>2.0 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>(10)</td>
<td>(9)</td>
<td>(7)</td>
<td>(3)</td>
</tr>
</tbody>
</table>

* Mean tumour diameter (± s.e.m.) measured as described in Materials and Methods. Numbers in parenthesis refer to number of surviving animals.
† Indirect IFM and IARR were performed on sera obtained from animals from each group, at the point of death on the 16th day. The sera were twice absorbed with lymphoid cells from 1- to 2-month-old pre-leukaemic mice and assayed at a 1/2 dilution. Results are expressed as a percentage of target cells showing a fluorescent 'ring' reaction or rosette formation.
‡ Treated refers to viable leukaemic cells incubated with neuraminidase (12.5 units/2 × 10⁷ cells at 37°C for 30 min) prior to assay.
¶ This group of AKR mice was injected with RPMI 1640 in a volume equivalent to that of suspensions of the irradiated leukaemic cells.
¶¶ N.D., not determined.
AKR leukaemia cell glycoproteins

surface of the leukaemic cells. Interestingly, we found that the indirect IFM assay performed with pre-immune serum or direct IFM on leukaemic cells in EBSS gave negative reactions, whilst in the IARR approximately 10% of the leukaemic cells tested showed a positive reaction when tested with preimmune serum. A DARR carried out on the leukaemic cells produced a similar value, indicating a population of cells carrying endogenous surface immunoglobulin. The lack of a positive reaction with direct IFM is explicable in the light of the work of Haegert, Hurd & Coombs (1978), who made a comparison of the DARR with direct IFM in the detection of surface membrane IgG on human peripheral blood lymphocytes and showed the DARR to be more sensitive.

Whilst we concur with the finding of Okazaki et al. (1976) that the percentage of immunofluorescent cells is not affected by absorption of the serum from hybrid mice by foetal cells, it should be pointed out that these authors do not appear to have considered the possibility that their results may have been complicated by the presence of Fc-receptor-bearing cells. In this study we have been able to show, using the rosetting assay of Hallberg et al. (1973), that no more than 13(±2.5)% (11) of our leukaemic cell population carried Fc receptors. Populations that had been depleted to 3-5% of cells bearing Fc receptors, by the DARR followed by centrifugation on a Ficoll/Isopaque barrier, still showed a positive IARR (20%) when tested with antiserum taken from hybrid mice after the seventh inoculation. Alexander (1982) has criticized the lack of attention given by some investigators to the presence of normal cells within a tumour. We have subjected suspensions of leukaemic cells, prepared from subcutaneous implants in AKR mice, to either filtration on nylon wool or incubation with carbonyl iron filings followed by magnetic separation to remove surface immunoglobulin-bearing cells and phagocytes, respectively. Neither treatment altered the level of cells exhibiting a positive DARR (10%). It is unlikely therefore that these latter cells are monocytes, and since their level of surface immunoglobulin is below that detectable by direct IFM it is possible that these may be null cells (Chess, Levine, Macdermott & Schlossman, 1975).

Cell-surface labelling by the galactose oxidase/tritiated borohydride technique has been shown to be a valuable tool for the identification of glycoproteins and glycolipids of a variety of cell types (Gahmberg & Hakomori, 1973, 1975), and has been used to characterize the surface glycoproteins of mouse lymphoid cells (Gahmberg et al. 1976), and to compare the surface glycoprotein patterns of normal and malignant lymphoid cells (Andersson et al. 1977). The analysis of surface glycoproteins by this technique has also been offered as a new diagnostic tool for the classification of human leukaemias (Andersson et al. 1979). It was therefore particularly interesting that treatment of the leukaemic cells with galactose oxidase, which oxidizes D-galactosyl and N-acetyl-D-galactosaminyl residues at non-reducing terminals to carbon-6 aldehydes (Avigad, Amaral, Asensio & Horecker, 1962), destroyed the ability of the antisera prepared in hybrid mice to react with these cells, and that mild treatment with the reducing agent NaBH₄ (back to galactose/N-acetylgalactosamine) reverses this effect. It would appear, therefore, that certain free terminal non-reducing galactose (and N-acetylgalactosamine) residues are important determinants
in the antibody–antigen reaction. Treatment with neuraminidase, which will expose further galactosyl residues as evidenced by the increased binding of biotinylated RCA$_{120}$, enhances the reaction with antibody, though this could be partly the result of a decrease in surface charge (Cook & Jacobson, 1968) as opposed to the exposure of specific galactosyl residues.

We have made use of the fact that the reaction between leukaemic cells and antibody raised in hybrid mice is reversibly inhibited by treating the cells with galactose oxidase and borohydride to characterize the glycoconjugates involved in this reaction. Immunoprecipitation and PAGE of cells labelled via $[^3H]NaBH_4$ after treatment with neuraminidase and galactose oxidase demonstrates that a group of glycoproteins with apparent molecular weights $>70000$ is involved. Control experiments with anti-H-2/K serum resulted in the labelling of a major component of 47 000 and another of 35 000; an estimated molecular weight of 47 000 for the NP40–H-2K$^d$ complex solubilized in SDS has been obtained by Schwartz, Kato, Cullen & Nathenson (1973). Okazaki et al. (1976) have suggested that the biochemical isolation and characterization of leukaemia-specific surface antigens may determine whether these antigens are expressed by the cellular or the viral genome. It is tempting to suggest that the glycoproteins detected here are precursor polyproteins of the murine leukaemia virus (Tung, Yoshiki & Fleissner, 1976; Ledbetter, Nowinski & Emery, 1977; Tung, Pinter & Fleissner, 1977). Serum from hybrid mice that gives an indirect immunofluorescence reaction with 78% leukaemic cells also reacts with various viral proteins as demonstrated by the blotting technique. However, absorption of this serum with leukaemic cells reduces the immunofluorescence reaction to 3.7%, without appreciably affecting the pattern of reactivity towards viral proteins. Therefore, while the glycoproteins detected by us may not be viral antigens it might reasonably be concluded that they are normal high molecular weight lymphoid glycoproteins (Gahmberg et al. 1976) with altered glycosylation patterns that are induced when the viral genomes are expressed.

The preparation of highly specific antibodies against leukaemia-specific surface antigens should provide a selective attack on transformed malignant cells (Okazaki et al. 1976). Antibodies in non-leukaemic AKR mice to the glycoproteins described by us can be raised by immunizing the animals with irradiated leukaemic cells either untreated or treated with neuraminidase. Neuraminidase-treated cells were included in this study in case sialic acids are masking cell-surface antigens (Jeanloz & Codington, 1976), and their removal results in enhanced antigenicity. However, the progression of tumour growth is not abated when viable leukaemic cells are introduced into either group of immunized animals. This may be due to the low levels of antibody produced, and though the titre might be increased by repeated immunization this approach seems to be unrewarding in that the host appears to be making antibodies directed against components that are also present in non-leukaemic cells.
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REFERENCES


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