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Antigenic Content of an *in vitro* Cellular Immunity Reactive Cell Surface Extract of Raji Cells

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With 2 Figures

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Summary

Antigenic content of a Raji cell surface extract (TS) was studied by sequential absorption of rabbit antiserum to this extract with different cells. Anti-TS was first allowed to react exhaustively with human blood cells followed by HeLa cells and human diploid fibroblasts. The residual reactivity was studied by stepwise absorption with an EBV-negative B lymphoblastoid cell line, BJAB, and then with B1—19, a clone derived from EBV transformed BJAB cells. These processes delineated three kinds of reactivities in anti-TS having specificities for antigens on B blasts, EBV-determined cell surface antigen(s) and antigens that are exclusive to Raji cells, respectively. These results are compatible with the heterogeneous protein content of TS and with the results of our earlier *in vitro* cell-mediated immunity studies using a similar preparation of Raji cell TS.

Introduction

Svedmyr and Jondal (15) demonstrated the presence of cytotoxic effector cells in patients with infectious mononucleosis that are specific for cell surface antigenic determinants (LYDMA) present on B cell lines transformed by Epstein Barr Virus (EBV). Nkrumah *et al.* (12) using a cytotoxicity assay observed a Burkitt's lymphoma-associated cell-mediated immune response against Raji cells. These are B lymphoblastoid cells which harbour EBV genomes but are not normally permissive to lytic synthesis of the virus (2). Ng *et al.* (11) showed that a surface extract of Raji cells (TS) elicited a disease-associated cell-mediated immune (CMI) response in patients with nasopharyngeal carcinoma (NPC). As all three diseases are related to EBV [see Klein (6)], it would appear possible

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that the above described CMI might be specific for a common or related virus-determined antigen(s) that may be found on all EBV-transformed cells regardless if the cells are permissive to lytic EBV synthesis. It would be of particular interest if such antigen might be found on NPC cells which are epithelial cells harbouring EBV genomes (7, 17). Consistent with this possibility, extracts of NPC biopsies have likewise been shown to elicit an NPC associated CMI response (11). However, experimental approach using CMI techniques does not readily allow detailed studies of antigenic specificity. Therefore, we have produced rabbit sera against Raji cell TS similar to that used for our earlier CMI studies. By sequential absorption of the resulting anti-TS with different cells, we report in this paper the detection of at least three kinds of antigens in the TS including one that may be EBV-determined. These results are in line with those of our earlier CMI studies and the availability of xenoantibodies to the TS now permits isolation of substances that are identical to, or crossreacting with, the TS antigens from different sources including NPC cells.

Materials and Methods

The Raji cell surface extract, TS, was prepared by treating these cells with a Tris HCl buffer (Tris HCl, 0.02 M, pH 7.2, containing 10 per cent glycerol) followed by centrifugation at $12{,}000 \times g$ for 30 minutes as previously described (8). To obtain the cytoplasmic fraction, Raji cells were homogenized in hypotonic buffer (Tris HCl, 0.02 M, pH 7.4) using a glass homogenizer with a teflon pestle (0.15 mm clearance). The homogenate was adjusted to contain 0.15 M NaCl and centrifuged at $100{,}000 \times g$ for 2 hours. All operations were performed at 4° C and the resulting supernatant is referred to as cytoplasmic fraction.

To prepare antisera, New Zealand white rabbits were immunized intramuscularly at weekly intervals with 1 mg proteins of TS or the cytoplasmic fraction. Serum antibodies to Raji cell surface usually reached a titer of 1:1000 or greater after the third injection. The animals were bled by heart puncture and the sera were stored at —20° C until use.

Indirect Immunofluorescent Test

Membrane immunofluorescence (MIF) was tested using $1—3 \times 10^6$ live Raji cells from log phase cultures. Hanks' buffer salt solution (HBSS) supplemented with 1 per cent fetal bovine serum was used throughout for washing of cells and dilution of antisera. The cells were washed twice and reacted at room temperature for 1 hour with a 0.2 ml aliquot of rabbit serum. The cells were washed 3 times, counterstained with the fluoresceine conjugated (FITC) goat anti-rabbit immunoglobulin for 1 hour and again washed. They were suspended in 0.1 ml of mounting solution (BSS in 50 per cent glycerol) and examined with a fluorescent microscope.

In some instance, antibody activity was assessed concurrently by agglutination of Raji cells. This is achieved visually immediately before the addition of FITC-anti-rabbit immunoglobulin by suspending the cells in 1 ml of the washing solution.

Antibody to intracellular components was tested by indirect immunofluorescence using acetone fixed smears of Raji cells. Raji cells were washed twice with phosphate buffered saline and resuspended in the same buffer to an approximate cell density of 2×10^7 cells per ml. A drop of the cell suspension was placed on a glass slide, thoroughly air-dried and fixed in chilled acetone for 8 minutes. The smears were allowed to react consecutively for 1 hour each with an aliquot of diluted rabbit serum and the FITC-anti-rabbit immunoglobulin with thorough washings after each step. The slides were mounted and examined in a fluorescent microscope. Antibody activity is expressed as the maximum reciprocal dilution which gave a positive reaction with the appropriate cell preparations.

Transformation of Cord Blood Leukocytes

Neonatal blood leukocytes were isolated by the method of BOYUM (1) using ficol isopaque gradient (lymphoprep, Oslo, Norway). To further minimize contamination of the isolated leukocytes with red blood cells, the cells harvested from the interphase were suspended in 0.81 per cent NH_4Cl for 30 minutes at room temperature, washed 3 times with RPMI-1640 supplemented with 15 per cent fetal bovine serum (Grand Island Biological Co., U.S.A.) and incubated in the same medium at a cell density of $1—2 \times 10^6$ cells per ml for 24 hours.

Culture supernatant of B95-8 cells which served as a source of EBV, was filtered through a millipore filter (0.45 μ) and stored in aliquots at —70° C until use. Leukocytes were allowed to absorb with EBV for 4 hours, washed, resuspended in culture medium (RPMI-1640 supplemented with 15 per cent fetal bovine serum) to a density of 0.5×10^6 cells per ml and incubated at 37° C in 5 per cent CO_2. Uninfected control cultures were similarly set up in parallel. About one half of the culture fluid was replaced with fresh medium once every week. Transformation is apparent by a) culture becoming rapidly acidic, b) appearance of abnormal lymphoblasts, c) clumping of cells (5, 10). This normally occurred within 6 weeks after infection. EBV-transformation is confirmed by the EBV-determined nuclear antigen (EBNA) test as described by REEDMAN and KLEIN (14).

Lymphoblastoid Cell Lines

The lymphoblastoid cell lines, Raji, Ly38, P_3HR-1, B95-8, and BJAB were obtained from Dr. G. de-Thé, QIMR-WIL from Dr. John Pope and B1—19 from Professor H. zur Hausen. These cells were cultured as suspensions in RPMI-1640 supplemented with 10 per cent fetal bovine serum.

Human Blood and Sera

The human blood used for absorption of the rabbit sera was obtained from the blood bank. It contained pooled cells from human blood groups A, B, AB and 0. The blood cells were fixed with 0.1 per cent glutaraldehyde for 30 minutes, washed with PBS containing 0.05 M glycine and kept resuspended in the same buffer at 4° C until use.

Results

The TS prepared in the same manner as used in our earlier *in vitro* CMI studies (11) and a cytoplasmic fraction of Raji cell were used to immunize rabbits. Sera obtained from these animals before and after immunization were tested by indirect immunofluorescence (IF) against live and acetone fixed Raji cells (Table 1). Anti-TS agglutinated live Raji cells and gave an intense membrane immunofluorescent (MIF) staining of these cells. MIF titers of these sera were substantially higher than the corresponding titers against acetone fixed smears of Raji cells.

Table 1. *Reactivities of hyperimmune rabbit sera against Raji cells*

Sera[a]	Animal	Titers		
		MIF	Agglutination	Cytoplasmic
Anti-TS	1	1620	540	160
Anti-TS	2	4860	540	160
Anti-Cytoplasm	3	40	10	640
Preimmune serum	1	10	10	10
Preimmune serum	3	10	10	10

[a] Antisera were produced in 3 New Zealand white rabbits against TS (rabbits 1 & 2) and the cytoplasmic fraction of Raji cells (rabbit 3)

By contrast, antiserum to the cytoplasmic fraction reacted more readily against acetone fixed smears than live Raji cells. These results indicated that anti-TS is reactive against the surface of Raji cells. The anti-TS is also reactive against the surface of different lymphoblastoid cell lines and freshly isolated human blood leukocytes (results not shown).

To further study membrane reactivity, anti-TS was allowed to react repeatedly first with glutaraldehyde fixed human blood cells followed by HeLa cells and human diploid fibroblasts and the residual activity of the absorbed sera was assayed against live Raji cells. The results obtained from 5 similar experiments were averaged and expressed as geometric mean titers (GMT). Figure 1 shows that absorption with blood cells did not cause a significant change in MIF titer of the sera. Neither were MIF titers affected appreciably by subsequent reaction with HeLa cells and human fibroblasts (results not shown). However as TS is a heterogeneous mixture of cell surface proteins (8) the apparently negative findings might have been anticipated because qualitative changes in seroreactivity due to this absorption may not be sufficient to cause a significant change in MIF titer against the homologous Raji cells. Nonetheless, antibodies to surface components of resting leukocytes appeared to have been absorbed. Thus when the absorbed sera were tested against freshly isolated peripheral leukocytes obtained from eleven neonates, only <1 per cent of cells from one leukocyte preparation gave a weak MIF reaction. However, after these cells had been infected with EBV, cultured for six weeks, and again similarly tested, cells from all the eleven transformed cultures gave intense MIF reaction against the absorbed rabbit serum (Table 2). Concomitant with the appearance of MIF reactivity, a substantial

Table 2. *Appearance of membrane immunofluorescence and EBNA in EBV-transformed neonatal leukocytes*

Neonatal leukocyte culture	Per cent reactive cells			
	Before infection		6 weeks after infection	
	MIF[a]	EBNA[b]	MIF	EBNA
C1	0	nd[c]	8.4	28.5
C2	<1% (weak IF)		8.2	23.5
C4	0	nd	7.5	36.5
C5	0	nd	20.5	38.0
C6	0	nd	9.0	50.5
C7	0	nd	5	40.5
C8	0	nd	5	55.5
C9	0	nd	5	46.0
C10	0	nd	9.6	49.5
C10N	0	nd	18.6	42.5
C11	0	nd	24.4	69.5

[a] Per cent membrane reactive cells in log phase cultures were determined in duplicate by indirect immunofluorescent technique against the 1:10 and 1:40 diluted rabbit sera to TS which had been exhaustively absorbed against glutaraldehyde fixed human blood cells, human diploid cells (FS 3) and HeLa cells

[b] Per cent EBNA positive cells in the cultures was determined according to the method of Reedman and Klein (1973)

[c] nd: not determined

proportion of these cells also gave positive staining of EB-determined nuclear antigens (EBNA) indicating that transformation had been mediated by the virus [see POPE (13)].

The above absorbed sera were allowed to react repeatedly with BJAB cells, a human lymphoblastoid cell line of B cell origin that does not carry EBV genomes (9). Typically, absorption with these cells resulted in an initial decrease in MIF titer of the serum presumably due to absorption of antibodies directing against B blast antigens (4, 16). Exhaustive absorption with BJAB cells, however, did not completely remove the reactivity against Raji cell surface, whereas when allowed to react similarly with the homologous Raji cells the MIF titer decreased to an undetectable level (Fig. 2).

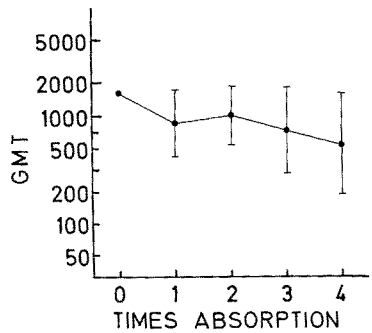

Fig. 1. Repeated absorption of anti-TS rabbit sera against glutaraldehyde fixed human blood cells. Five experiments have been performed. In each experiment, 1 ml anti-TS was repeatedly absorbed against packed human blood cells. Membrane IF titer was determined after each absorption and the results are expressed as the geometric mean titer (GMT). The standard deviation (SD), when applicable, is indicated by vertical bars

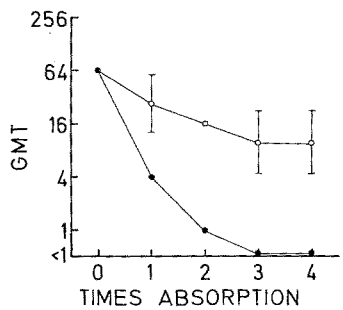

Fig. 2. Repeated absorption of anti-TS rabbit sera with Raji and BJAB cells. Rabbit anti-TS serum was first exhaustively absorbed with human blood cells, followed by HeLa cells and human diploid fibroblast. One ml of a diluted aliquot of the absorbed serum was repeatedly reacted against 10^7 Raji cells (•) or BJAB cells (○). MIF titer after each absorption was determined against live Raji cells and the results obtained from 3 such experiments were expressed as geometric mean titers. The standard deviation (SD), when applicable, is indicated by vertical bars

The BJAB-absorbed sera were further tested for membrane reactivity against different EBV-carrying lymphoblastoid cell lines of B cell origin including a clone of EBV-transformed BJAB cells, B1—19 (3). Table 3 shows that the BJAB absorbed serum was reactive against the membrane of all of the EBV-carrying lymphoblastoid cells tested. Reactivity against the EBV-transformed BJAB cells was studied by repeated absorption with B1—19 cells. The extent of absorption was assessed concurrently by counterstaining the reacted B1—19 cells with FITC anti-rabbit immunoglobulins and by the residual membrane reactivity of the absorbed sera against Raji and BJAB cells. Table 4 shows the results of a typical experiment which seems to have delineated two types of membrane reactivities in the BJAB absorbed serum. The initial decrease in MIF titer might be due to absorption of reactivity against an EBV-related antigen present on B1—19 cells. MIF titers of the serum remains essentially unchanged after the second absorption and the serum was no longer reactive against the surface of B1—19 cells after the fourth. It was considered likely that the residual reactivity in this exhaustively absorbed serum might be directed against antigens that are exclusive to Raji cells.

Table 3. *EBV-related membrane reactivity of rabbit antisera*[a]

Cell	EBNA	Per cent MIF cell ± SD
Raji	+	26.5± 9.4
Ly 38	+	16.8±16.5
QIMR-Wil	+	7.7± 5.0
P_3HR-1	+	19.1± 2.2
BJAB	−	Not detected

[a] Rabbit anti-TS serum was first absorbed exhaustively against human blood cells, followed by human diploid fibroblasts, HeLa cells and BJAB (cf. Fig. 2). The absorbed sera were allowed to react against live lymphoblastoid cells at 1:4 dilution and the MIF reactive cells out of a total of not less than 200 were counted. This experiment was repeated 3 times and the result expressed as mean per cent MIF cell ± SD

Table 4. *Absorption of anti-TS with EBV transformed BJAB cells*

Number of absorptions	Membrane reactivity[a]		
	B1—19	Raji (titer)	BJAB (titer)
0	+	64	<4
1	+	16	<4
4	+	16	<4
5	−	16	<4

[a] One ml of the BJAB-absorbed serum from Figure 2 was further absorbed repeatedly with 6×10^6 cells each of B1—19 cells which is a clone of EBV transformed BJAB cells. The reacted cells were counterstained with FITC-anti rabbit immunoglobulin and positive membrane reactivity indicated as +. MIF reactivity (titer) of the absorbed sera were determined concurrently against Raji and BJAB cells

Discussion

The antigens of the Raji cell extract, TS, have been analysed using rabbit antisera to TS. The results obtained by sequential absorption of the antisera with different cells revealed four kinds of reactivities directed respectively against antigens present on a) resting leukocytes, b) EBV-negative B lymphoblasts, i.e. BJAB, c) EBV-transformed lymphoblastoid cells including a clone derived from EBV infected BJAB cells, and d) antigens that are present exclusively on Raji cells. These results are compatible with the heterogeneous protein content of the Raji cell TS (8).

The present findings provide further support for the presence of an EBV-determined cell surface antigen(s) occurring on EBV-transformed B lymphoblastoid cell lines. Reactivity against these antigens in the rabbit anti-TS is defined operationally as that which is present in the BJAB cell-absorbed sera but which could be absorbed with EBV-transformed cell lines such as B1—19. As Raji cells are not normally permissive to EBV lytic synthesis, it is unlikely that such an antigen is a product of the lytic cycle of this virus. It would be of particular interest if this xenoantibody-defined EBV antigen(s) might be structurally related to LYDMA (15) and be also responsible, partly or wholly, for eliciting disease-associated *in vitro* CMI observed in patients with Burkitt's lymphoma (12) and NPC (11).

The present studies were undertaken in a continuing effort to elucidate the antigenic specificity of the NPC associated CMI observed with the extracts of Raji and NPC cells (11). With the availability of the xenoantibodies to TS, it is now possible to test whether the disease-associated CMI is due to crossreacting or identical antigens present in both types of extracts and if so, what the nature of such antigens is. We have therefore tested NPC extracts before and after absorption with the immobilized anti-TS against leukocytes obtained from NPC patients and control subjects. The immobilized anti-TS also allowed isolation and purification of the crossreacting antigens from the tumour extracts. Results of these studies will be reported later.

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