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Expression of murine H–2K^b histocompatibility antigen in cells transformed with cloned *H–2* genes

A. L. Mellor*, L. Golden*, E. Weiss*, H. Bullman*, J. Hurst*, E. Simpson†, R. F. L. James‡, A. R. M. Townsend§, P. M. Taylor§, W. Schmidt¶, J. Ferluga‖, L. Leben‖, M. Santamaria‖, G. Atfield‖, H. Festenstein‖ & R. A. Flavell*

* Laboratory of Gene Structure and Expression, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
† Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, UK.
‡ ICRF Tumour Immunology Unit, University College, Gower Street, London WC1E 6BT, UK
§ Division of Immunology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
‖ Department of Immunology, The London Hospital Medical School, University of London, Turner Street, London E1 2AD, UK

Cosmids containing H–2 *histocompatibility antigen genes of the* H–2^b *haplotype have been isolated. One of these genes expresses a 45,000 molecular weight protein, indistinguishable from* H–2K^b *when introduced into mouse L cells. These* H–2K^b *transformed L cells can be killed by allospecific anti-*H–2K^b *cytotoxic T cells. Moreover, when infected with influenza virus, they can be killed by an* H–2K^b*-restricted, influenza virus-specific cytotoxic T cell line. These results show that expression of the* H–2K^b *gene product on the L-cell surface is sufficient to make it a target for specific T-cell killing.*

CELL-surface glycoproteins encoded by the genes of the major histocompatibility complex (MHC), *H–2* in mouse, *HLA* in man, are important in regulating cell–cell interactions, especially those governing the functions of the immune system[1]. Class I molecules, of which the best characterized are encoded by genes on the H–2K, D and L regions (Fig. 1*a*) act as guides for presenting extrinsic antigen (for example viruses) to cytotoxic T cells, whilst class II molecules encoded by genes in the I-region perform a comparable function for T helper cells[1]. How extrinsic antigens are presented in association with self-MHC molecules is still very poorly understood, partly because the structure of the T-cell receptor is unknown. However the problem can be approached by study of MHC molecules themselves. Knowledge of the amino acid sequence and three-dimensional structure of these molecules has progressed very rapidly over the past few years (see, for example, ref. 2). The *H–2K* and *H–2D* gene products are highly polymorphic, which may reflect the importance of genetic variation of MHC molecules in overcoming virus infections. The mutation rate, particularly of class I H–2 molecules is extremely high[3] and of all of the *H–2* haplotypes studied, that of *H–2^b* is the highest. Within this haplotype it is the *H–2K^b* molecule which undergoes mutational change most frequently. Nathenson *et al.*[4] have determined the complete polypeptide sequence of the H–2K^b molecule of C57BL/10 mice and have also determined portions of the sequence of several mutants[5] which express an altered H–2K^b molecule. Information about the DNA coding for these molecules is thus of great interest, and may give insight into the mechanism of mutation and generation of new haplotypes.

Several groups have isolated cDNA[6,7] and genomic DNA clones[8–10] containing class I *H–2* or *H–2* related DNA sequences from BALB/c mice (*H–2^d* haplotype). We have isolated ~100 different cosmid clones, containing sequences mapping to the *H–2* and associated regions (Fig. 1*a*) of chromosome 17 from a cosmid library constructed using spleen DNA from C57BL/10 mice (*H–2^b* haplotype). A full description of the construction and screening of these libraries together with chromosomal mapping data for all the *H–2*-related cosmid clusters, will be given elsewhere. Here we describe a cluster of five overlapping cosmids (the H8 cluster) and show that one of the two *H–2* gene sequences cloned in this cluster encodes a cell-surface antigen which is recognised by *H–2K^b* restricted T cells. It therefore appears identical to the *H–2K^b* polypeptide found on cells derived from C57BL/10 mice.

Restriction map of the H8 cosmid cluster

The H8 cluster of cosmids consists of five overlapping cosmids containing two class I *H–2* related genes, approximately 15 kilobases (kb) apart, which hybridize to human genomic[11] and *H–2* cDNA class I gene probes[6] (Fig. 1*b*). The DNA cloned in this cluster spans about 65 (kb) of the C57BL/10 genome and the two *H–2* class I gene-related sequences are arranged in a head to tail configuration, as determined by hybridization to 5′ and 3′ probes obtained from the *HLA* or *H–2* probes. The left-hand gene (*H–2* genes are defined here as DNA sequences which hybridize to *HLA* and *H–2* class I probes) is present in cosmids H8, H25, H24 and H39 (A gene), whereas the right-hand gene sequence (B gene) is present only in H8 and H21. As polymorphic restriction site mapping of this cosmid cluster shows that the cloned region is derived from the *H–2K* (Fig. 1*a*) end of the *H–2* complex (manuscript in preparation), we investigated whether one (or both) of the two gene regions in the H8 cluster encoded an *H–2K^b* cell-surface antigen. Thus, we have introduced each cosmid into mouse Ltk$^-$ (of *H–2^k* haplotype) cells using $CaPO_4$-mediated DNA transfer and tested the resulting tk$^+$ clones for expression of new H–2^b cell-surface antigens using (1) direct monoclonal and allo-antibody binding (2) antibody-dependent complement-mediated lysis and (3) alloreactive anti-H–2K^b T-cell mediated lysis (CML) and (4) H–2K^b-restricted virus specific T-cell killing.

¶ Present address: Institut für Virologie, Frankfurter Strasse, 107, 6300 Gissen, FRG.

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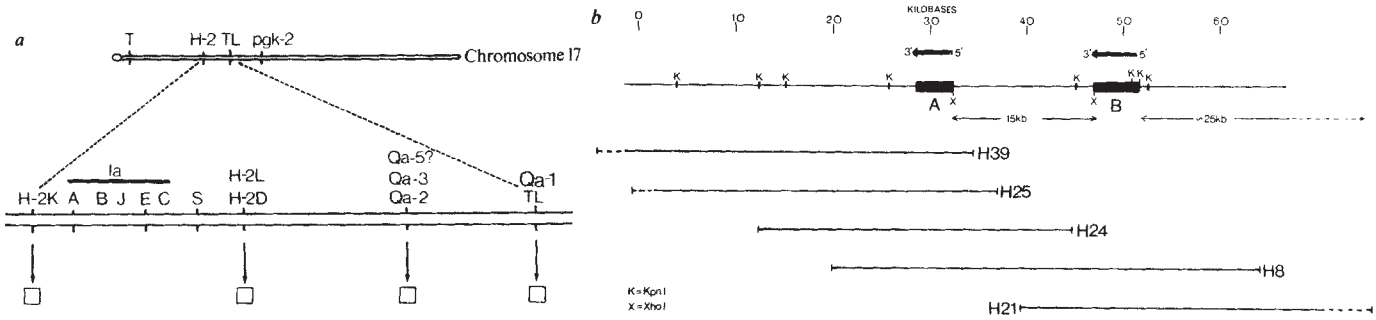

Fig. 1 *a*, Genetic map of the mouse *H-2* and associated genetic loci. The top line shows a diagram of chromosome 17 with the centromere on the left. The bottom line shows an expanded diagram of the regions between the *H-2K* locus on the left and the TL locus on the right. H-2 class 1 molecules (□) are expressed from at least four separate genetic loci as shown by the arrows below this line. *b*, A restriction enzyme map of the region cloned in the H8 cosmid cluster. The top line shows the extent of the cloned region and the approximate positions of *Xho*I(X) and *Kpn*I (K) restriction sites (see top scale calibrated in kilobase, kb). Class 1 gene regions (A and B) are shown as thick lines and their 5' to 3' orientation is indicated by the thick arrows over the line. The approximate extent of each cosmid is shown below the line beside the name assigned to each cosmid. Dotted lines at the end of cosmids indicate that the end of the cosmid has not been determined precisely.

Table 1 Antibody-dependent cytotoxicity assays

Antisera/antibodies	% Specific ^{51}Cr release (cell lines) Ltk$^-$	LH8	LH39
WS9/7B (αK^k)	73	71	61
D32(αD^k) NIH	63	71	61
D1 (αH-2^k) NIH	58	72	66
D8 (αH-2^k) NIH	21	70	44
100-5 (anti-H-2.11/αH-2K^k) monoclonal*	62	50	44
Anti-H-2.33 (αK^b) NIH unabsorbed	2	72	6
(Ia-9-20) B10 absorbed	0	0	0
AKR absorbed	2	66	0
D28 (αH-2^b) NIH unabsorbed	21	70	44
B10 absorbed	1	1	0
AKR absorbed	2	48	0
27-11-13 (αD^b)†	4	6	0

The chromium release assay was performed essentially as described by Sanderson[19]. Details of all antisera and monoclonal antibodies except 100-5[20] and 27-11-13[21] can be found in ref. 14. Absorptions of antisera were carried out before binding to cell lines using either C57BL/10 (B10) or AKR lymphocytes. Cell lines tested for lysis in this experiment were untransformed L cells (Ltk$^-$) and uncloned tk$^+$ cells transformed by cosmid H8 (LH8) or cosmid H39 (LH39). % Specific ^{51}Cr release was measured as (test c.p.m. − spontaneous c.p.m./maximum c.p.m.) × 100. Spontaneous release was 10–13%. Numbers shown in a box are significant results showing specific H-2K^b expression.

* Lemke *et al.*[20].

† Ozato and Sachs[21].

Introduction of cosmid DNA

The cosmid library used in this study was constructed using the cosmid vector pOPF1 (F. Grosveld *et al.*, in preparation). As this vector contains the herpes simplex virus thymidine kinase (*tk*) gene, cosmid DNA can be selected for its presence in transformed L cells by growing the cells in hypoxanthine/aminopterin/thymidine (HAT) medium. Thus, the five cosmids from the H8 cluster were used to generate tk$^+$ clones following $CaPO_4$-mediated DNA transfer and subsequent selection for tk$^+$ clones in HAT medium[12]. For each cosmid transformation, two subclones were isolated and the rest of the tk$^+$ clones were passaged as an uncloned tk$^+$ population. We experienced some difficulty with cosmid H21, due to its propensity to lose DNA sequences whilst replicating in host bacteria.

Analysis of transformed L-cells

Binding of antibodies: L cells transformed by individual cosmids and established as stable Ltk$^+$ transformed clones growing in HAT medium, were tested for their ability to bind anti-H-2K^b or anti-H-2D^b monoclonal antibodies in ^{125}I-radioimmunoassays. The results of a binding assay using Ltk$^+$ cell clones derived by transformations with H8, H24, H25, H39 and a control, H11 (a cosmid which maps to the H-2 related TL region; in prepa ation) are shown in Fig. 2. Both clones derived from H8 transformations (LH8.1 and LH8.2) bind anti-H-2K^b monoclonal antibody at a level higher than that of the positive control EL4 cell line (an H-2^b fibrosarcoma cell line). All other cell lines tested show only background levels of anti-H-2K^b monoclonal antibody binding. Anti-H-2D^b monoclonal antibody was not bound to any of the cell lines tested. Recently, Goodenow *et al.*[13] and Evans *et al.*[10], have both detected the expression of a cloned *H-2L^d* gene in transformed cells by using a monoclonal antibody binding assay.

In addition, a series of well characterized anti-H-2 alloantisera was used. In this case, binding of antibodies to L-cell lines was assessed using either complement-dependent chromium (^{51}Cr) release (Table 1) or complement-mediated growth inhibition assays (Table 2).

These assays both demonstrate that L cells transformed with cosmid H8 express a cell-surface polypeptide which is recog-

Table 2 Antibody-dependent growth inhibition

Antisera/antibodies		% Specific growth inhibition cell lines L cells	LH8.1	LH39
Anti-H-2.23 (αK^k) NIH		74.8	85.8	87.3
100-30 (anti-H-2.25/αK^kD^k) monoclonal*		78.8	89.6	93.5
Anti-H-2.28 (αH-2^b)	NIH unabsorbed	4.2	66.2	7.1
	B10 absorbed	NT	6.3	3.9
	AKR absorbed	NT	40.5	0
Anti-H-2.33 (αK^b)	NIH unabsorbed	0	86.4	0
	B10 absorbed	NT	0	0
	AKR absorbed	NT	86.9	0

NT, not tested. For sera see Table 1. B10 = C57BL/10. The growth inhibition assays were performed as described previously[22]. L cells were cultured in Cooke flat-bottomed microculture plates at 10^4 per well in 4 μl for 45 min at 37 °C in RPMI with 10% fetal calf serum in CO_2 and air with 1 μl of antibody per well (final dilution 1/5). Rabbit complement (1/5 diluted) was then added and the incubation continued for another 30 min. ^{14}C-uridine was added and the incubation continued for 6 h. 0.025% Trypsin in phosphate-buffered saline was then added and incubated for 30 min. After three washes the wells containing adherent cells were punched out and counted in a scintillation counter. Cell lines tested in this assay were untransformed L cells (Ltk$^-$), a cloned L-cell line transformed by cosmid H8 (LH8.1) and uncloned tk$^+$ cells transformed by cosmid H39 (LH39). Numbers shown in a box are significant results showing specific H-2K^b expression.

* Lemke *et al.*[20].

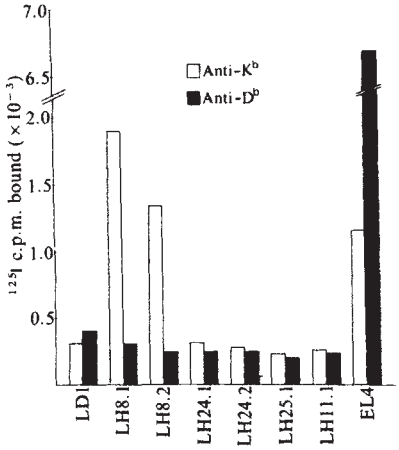

Fig. 2 Histogram showing the binding of monoclonal antibodies to transformed L cells. Binding studies were done in Titertek 96 well flexible plastic plates (Flow Laboratories). These were first blocked with radioimmunoassay RIA buffer (0.1 M phosphate-buffered saline, *p*H 7.2 plus 0.02% NaN_3 + 1% bovine serum albumin) and cells were assayed at 5×10^5 per well in triplicate. Cells were incubated with the first antibody in a volume of 100 μl of MEM–H (Eagle's minimal essential medium plus HEPES buffer +0.1% NaN_3 + 5% calf serum) for 1 h at room temperature on a Titertek plate shaker (Flow), washed 3× with MEM–H and then incubated for 45 min with ^{125}I-labelled affinity purified sheep anti-mouse immunoglobulin (10–15 μCi per μg) in a volume of 100 μl containing ~10^5 c.p.m. per well. Cells were washed 3X and counted in a Nuclear Chicago γ scintillation counter. The monoclonal antibodies used were anti-H–2K^b(Y25, a gift of E. A. Lerner) used at a dilution of 1:2,000 or anti-H–2D^b (B22–249–R1, see ref. 23) used at a dilution of 1:100. LD1 cells are Ltk$^-$ control cells.

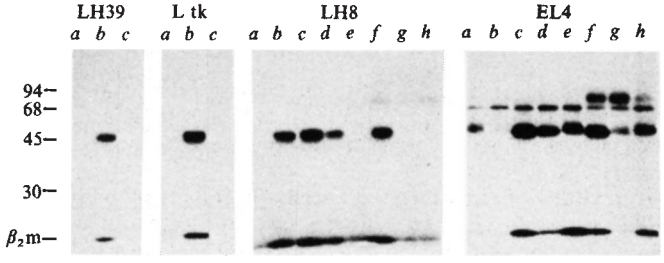

Fig. 3 Immunoprecipitation of radiolabelled cell-surface antigens from transformed L cells. Cell-surface proteins of cells indicated were radiolabelled with ^{125}I by the glucose oxidase–lactoperoxidase catalysed iodination technique[24] and lysed with nonionic detergent (0.5% NP40 in 0.01 M Tris-HCl buffer, *p*H 7.4, containing 0.15 M NaCl, 1.0 mM $MgCl_2$) in the presence of 1.000 KIE ml^{-1} Trasylol (soy bean trypsin inhibitor). Reaction of solubilized cell membrane proteins with monoclonal antibodies and alloantisera was determined by immunoprecipitation using *Staphylococcus aureus* and SDS-polyacrylamide gell electrophoresis of the resulting precipitates as previously described[25]. Precipitations were done with BALB/c–normal mouse serum (control) *a*, with monoclonal antibodies (refs. 19, 20) specific for H–2K^k (H100–5) *b*; H–2K^b (20–8–4s) *c*; H–2K^b, D^b (28–8–6s) *d*; H–2D^b, L^d(28–14–8s) *e*; and with alloantisera raised against the private specificities of H–2K^b *f*, *g*; and H–2D^b *h*. The anti-H–2K^b sera (A×B10.D2)F$_1$ anti-B10.A (5R)) were either preabsorbed with AKR (H–2^k) lymphocytes which remove antibody against murine leukaemia virus gp70 but not H–2 (*f*), or with B10 lymphocytes, which specifically remove anti-H–2K^b alloantibody (*g*). Note that both monoclonal antibodies and alloantiserum with specificity for the H–2K^b molecule precipitated a 46,000 molecular weight protein, which is identical in molecular weight to the H–2K^b expressed on EL4 (*H*–2^b) tumour cells. Co-precipitation of β_2-microglobulin (β_2m), incorporation of trititated mannose and glucosamine, and binding to *Lens culinaris* Sepharose (not shown) indicate that the cloned *H*–2K^b gene product is expressed on L cells in the same manner as in normal *H*–2^b lymphoid or tumour cells (ref. 4).

nized by public (anti-H–2.28) and private (anti-H–2.33) specificity alloantisera[14] reactive against the H–2K^b molecule. Antibody binding to these cell lines was shown to be specific for H–2^b antigens, because preabsorption of the antisera with spleen cells from B10, but not AKR (*H*–2^k) mice blocks binding. L cells transformed with cosmid H39, which contains the A gene, but not the B gene, do not express the public or private *H*–2^b determinants defined by the alloantisera we used. All cell lines, including untransformed Ltk$^-$ cells (which are derived from the C3H mouse of *H*–2^k haplotype) react with monoclonal antibodies and alloantisera directed against H–2^k cell-surface antigens. The data suggest that cell lines transformed with cosmid H8 express a cell-surface antigen which is indistinguishable from the H–K^b molecule using these antibodies. In addition, the lack of any significant anti-H–2K^b antibody binding

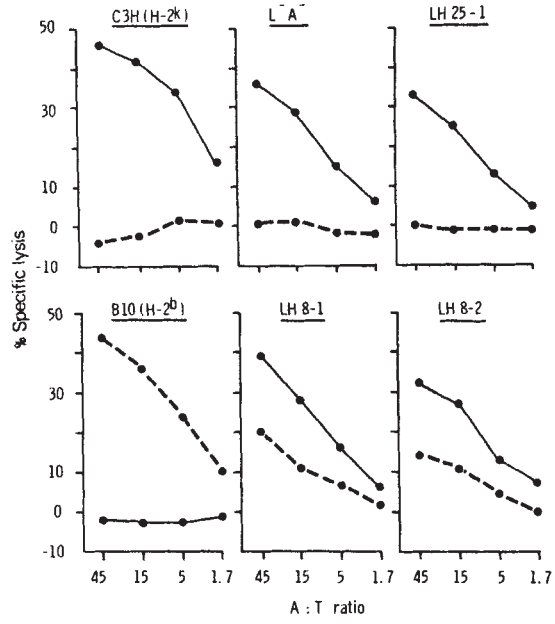

Fig. 4 1–2×10^6 target cells were labelled with 100 μCi ^{51}Cr-labelled sodium chromate for 1 h then washed twice. 1×10^4 Labelled target cells, in 100 μl volumes were placed in round-bottomed wells of microtitre plates. For the cytotoxic T-cell lysis (CML) killer cells, generated in 5-day mixed lymphocyte cultures (CBA anti-BALB.B = k anti-b, B10 anti-CBA = b anti-k) were added in 100 μl to give attacker: target (A:T) ratios of 45:1, 15:1, 5:1 and 1.7:1. Three triplicate wells of each A:T ratio were set up. After 5 h incubation at 37 °C in a 5% CO_2 humidified atmosphere the plates were spun and 100 μl of the supernatant from each well collected and counted for released γ counts. The per cent specific lysis was calculated according to the formula $(E-C/(M-C)\times100\%$ where E = c.p.m. from target cells incubated with killer cells, C = c.p.m. from target cells incubated in medium alone and M = c.p.m. from target cells lysed with 5% Triton. L$^-$A$^-$ refers to control Ltk$^-$ cells.

to those cell lines transformed with cosmids H24, H25 and H39 suggests that the H–2K^b polypeptide detected in H8 transformed cells is expressed from the B gene in the cloned region.

Detection of H–2K^b in the L-cell membrane: Confirmation that L-cells transformed with cosmid H8 contain H–2K^b heavy chain polypeptides on the cell surface was obtained by labelling the cell surface proteins of transformed cells with ^{125}I and immunoprecipitating the cell extracts. This was done by using either alloantisera o monoclonal antibodies directed against the H–2K^b molecule. Immunoprecipitates were analysed by SDS–polyacrylamide gel electrophoresis and the results are shown in Fig. 3. The H–2K^d-specific alloantisera and two monoclonal antibodies (anti-H–2K^b and anti-H–2K^{b}D^b) precipitate cell-surface polypeptides of molecular weight 45,000 and 12,000 (H–2 heavy chain and β_2-microglobulin respectively) from extracts of L cells transformed with cosmid H8. No polypeptides were precipitated from the LH8 cells by the anti-H–2D^b/L^d-specific monoclonal antibody (Fig. 3, lane *e*).

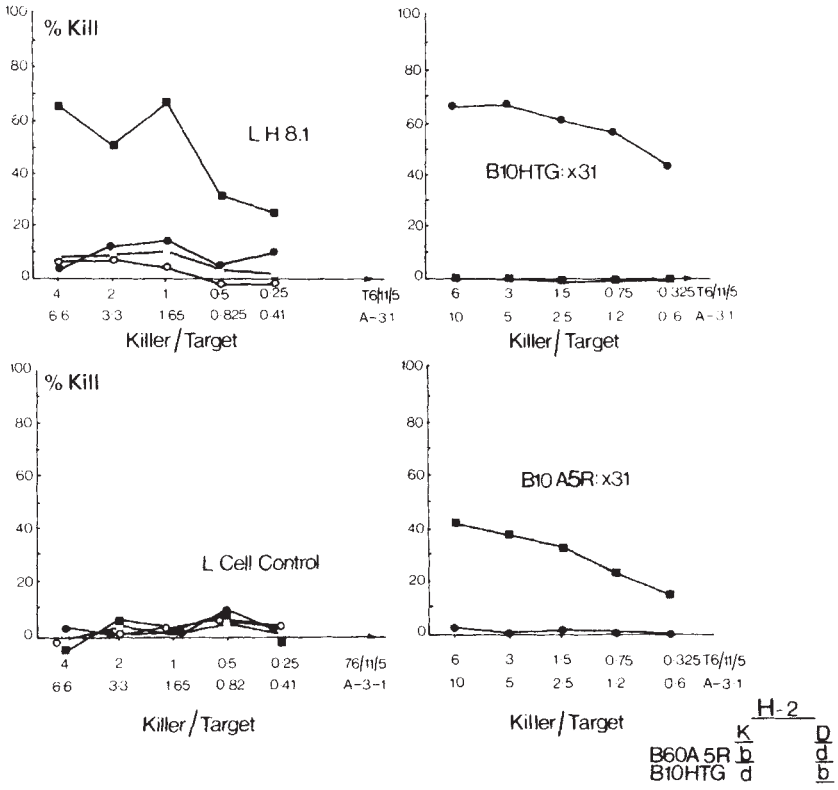

Fig. 5 T_c-cell mediated killing of transformed L cells infected with influenza virus. T_c-cell clones: The derivation of the two influenza-specific T_c clones T6/11/5 and A3.1 will be described in detail elsewhere (A.R.M.T. and P.M.T., manuscript in preparation). Clone A3.1 is restricted to H-2D^b and clone T6/11/5 to H-2K^b. In brief, C57BP6 mice were primed by ultranasal infection with A/X31 or A/JAP influenza virus. T_c-cell lines were established from spleen cell suspensions from these mice by repeated stimulation with 2,000R irradiated A/X31 infected syngeneic spleen cells, either in the presence (A3.1) or absence (T6/11/5) of a source of growth factors from concanavalin A-pulsed rat spleen. Cells from both sources were cloned at 0.5–10 cells per well onto 5×10^5 2,000R X31 infected syngeneic spleen in the presence of 10–20% concanavalin AS/N. 10–20 days later they were transferred to 2-ml wells and maintained by repeated antigenic stimulation with 2,000R X31 infected syngeneic spleen in the presence of 20% concanavalin AS/N every 5–10 days. Chromium release assay: Transformed and untransformed L cells were plated out at 3×10^4 per well in 100 μl α medium containing 10% fetal calf serum 18 h before assay into Nunc 96 well flat-bottomed wells plated in the presence of 2 μCi of sodium chromate (^{51}Cr). Before the assay the plates were spun at 1,000 r.p.m. for 5 min and the medium from wells containing cells to be infected removed by suction. To each of these wells was added 100 μl of serum free α medium containing 320 HA A/X31 and the plates incubated for a further 1.5 h. The plates were then washed four times with 200 μl per well balanced salt solution and the cells in all wells finally resuspended in 100 μl of RPMI medium plus 10% fetal calf serum. Control target cells were 3% thioglycollate-induced peritoneal exudate cells from B10A5R (*H-2K^b, D^d*) and B10 HTG (*H-2K^d, D^b*) mice. 10^7 cells were labelled with 100 μCi Na chromate (^{51}Cr) and infected with 3,200 HA A/X31 in 0.5 ml serum free RPMI for 1.5 h. Immediately before the assay they were washed four times in 10 ml phosphate-buffered saline and resuspended in RPMI 10% fetal calf serum. 2×10^4 targets were dispensed in 100 μl to wells of Nunc 96 well flat-bottomed microtitre plates. The cloned cytotoxic cells, at the ratio indicated in the figure, were added in 100 μl plus 10% fetal calf serum to experimental wells, 100 μl RPMI plus 10% fetal calf serum alone was added to control wells. The plates were spun at 1,000 r.p.m. for 1 min and then incubated for 6 h at 37 °C. The collection of ^{51}Cr-containing supernatants was done as previously described[26]. All samples were counted on a Wallac 80,000 γ counter for 2 min. Results were calculated as % specific ^{51}Cr release from target cells as follows:

$$\frac{\text{release in presence of cloned } T_c - \text{medium release}}{\text{Triton release} - \text{medium release}} \times 100\%.$$

All points are the mean from three identical wells. ○, Control target cells; A-3.1 H-2D^b (restricted) effectors. □, Control target cells; T6/11/5 (H-2K^b restricted) effectors. ●, Infected target cells; A-3.1 effectors. ■, Infected target cells; T6/11/5 effectors.

Anti-H-2K^b antibodies also fail to detect any polypeptides in untransformed Ltk$^-$ cells or in cells transformed with cosmid H39 which contains the A gene. Preabsorption of the anti-H-2K^b alloantisera using unlabelled B10, but not AKR lymphocytes prevents the precipitation of H-2K^b and β_2-microglobulin from extracts of cells transformed with cosmid H8 (Fig. 3, lanes *g* and *f* respectively).

Cytotoxic T-cell killing of L-cells: The presence of the H-2K^b molecule on a cell renders these cells as targets for allogeneic cytotoxic T-cell (T_c-cell)-mediated killing by T_c cells raised against H-2^b antigens[15]. A number of indirect arguments suggest that the presence on the cell membrane of a given H-2 polypeptide, such as the H-2K^b molecule is sufficient to generate a target for T_c-cells. For example, this process can be blocked by monoclonal antibodies directed against a single H-2 polypeptide[16]. To test this further, we generated T_c cells directed against H-2^b or H-2^k antigens and tested their ability to kill various target cells (Fig. 4, Table 3). Whereas anti-H-2^k T_c cells show specific killing of all L-cell lines tested, anti-H-2^b T_c-cells show specific killing of only control B10 target cells and two L-cell clones (LH8-1 and LH8-2) transformed by cosmid H8. Untransformed L cells (Ltk$^-$), a clone transformed by cosmid H25 (LH25-1) and other clones transformed by cosmids H24 and H39 (data not shown) were not killed by anti-H-2^b T_c cells. These data confirm that the H-2K^b molecule detected by serological assays in cosmid H8 transformed cells, can act as a target for anti-H-2^b T_c-cell killing.

We performed a similar experiment using B10.D2 (*H-2^d*) Tc cells generated by immunization with target spleen cells from the recombinant mouse B10.A (5R) *K^b, D^d*). Such T_c cells kill cells expressing the H-2K^b but not the H-2D^b molecule. These Tc cells also kill L cells transformed with cosmid H8, but not L cells transformed with cosmid H39 (Table 3).

Thus, these data suggest that the B gene present in cosmid H8, but not present in cosmids H24, H25 and H39, encodes a molecule carrying the determinant recognized by anti-H-2^b and anti-H-2K^b T_c cells.

T-cell killing of virus-infected L-cells: T_c cell mediated lysis of cells expressing viral antigens on their surface can only take place when the viral antigen is presented to T_c cells in association with an appropriate H-2 antigen; in this way H-2 antigens act as a restriction element for the T_c-cell response[15,17]. Thus, we tested the ability of the H-2K^b molecule expressed on the surface of L cells transformed by cosmid H8 to act as a restriction element for T_c-cell-mediated killing of influenza virus-infected cells.

Influenza-specific T_c clones restricted to one H-2 region can be selected and grown in the presence of T-cell growth factor[18]. L cells (*H-2^k*) and LH8-1 cells were infected with type A influenza virus (A/X31) and used as target cells for two influenza-specific H-2^b T_c clones, one restricted to H-2K^b (T6/11/5) and the other (A/3.1) to H-2D^b molecules (A. Townsend and P. M. Taylor, unpublished data). Figure 5 shows that X31 infected, untransformed L-cells (H-2^k) are unable to act as targets for killing by either the H-2K^b or H-2D^b restricted T_c-cell clones. LH8-1 cells infected with X31, however, act as targets for killing by the H-2K^b restricted T_c-cell clone, but not the H-2D^b restricted clone. This shows that the H-2K^b molecule expressed on the surface of LH8-1 cells is able to act as a restriction element for T_c-cell killing of X31 virus-infected cells.

Discussion

We have shown here that the H8 gene cluster contains two *H-2* genes, one of which (gene B) is an *H-2K^b* gene. The second gene (gene A) does not express a polypeptide that can be detected by any o the immunological criteria we have used

Table 3 Cell-mediated cytotoxicity

Responder strain (*H-2* haplotype)	Stimulator strain (*H-2* haplotype)		(Target cells)	% Specific lysis at effector: target ratio 100	50	10	1
B10.BR ($H\text{-}2^k$)	C57BL/10 ($H\text{-}2^b$)		L cells	7.0		1.2	−0.6
			LH8	52.7		28.9	3.6
		Expt 1	LH8.1	58.7		39.2	7.8
			LH39	6.0		4.5	1.4
			LH39.1	1.4		0.7	0.3
			LH8.1	45.0		19.1	2.6
			LH8.2	40.9		16.6	2.2
		Expt 2	LH39.1	7.1		1.9	1.8
			B10.blasts	59.5		48.6	18.0
			B10.BR blasts	8.0		8.1	3.2
B10.D2 ($H\text{-}2^d$)	B10A(5R) (*bbkkdddd*)		L cells		11.0	12.7	0.7
			LH8		51.7	26.0	4.1
		Expt 1	LH8.1		53.3	32.0	3.9
			LH39		22.0	10.3	2.8
			LH39.1		18.8	8.0	1.6
			LH8.1	50.3		25.3	3.8
			LH8.2	50.8		21.6	2.4
			LH39.1	16.4		5.3	0.7
		Expt 2	B10 blasts	53.4		42.0	9.0
B10A(5R)			46.2				
			41.2				
			10.0				
			B10.D2	16.4		10.1	3.2

Cytotoxic T lymphocytes (CL) were generated *in vitro* from splenic lymphocytes of responder strain mice (B10.BR and B10.D2) which had been inoculated 1 week earlier with 4×10^7 stimulator strain (B10 and B10.A(5R) respectively) splenic lymphocytes. Responder lymphocytes were cultured in flasks at 2×10^7 per ml with an equal number of irradiated (2,000 rad) stimulator splenic lymphocytes in 10 ml culture medium (RPMI 1640 with 10% fetal calf serum, 100 U ml^{-1} penicillin, 10 μg ml^{-1} streptomycin, 5×10^{-5}M 2-mercaptoethanol). Cells were collected after 5 days' incubation at 37 °C in 8% CO_2 in air and assayed for cytotoxic activity against normal (L cells) or transfected (LH8, LH39) tumour cells labelled with ^{51}Cr-labelled sodium chromate. These cells were grown as monolayers and detached by treating for 5 min with 0.25% trypsin/versene in phosphate-buffered saline before being labelled with sodium chromate (100 μCi per 5×10^6 cells) for 2 h, washed four times to remove any free radioactivity, then suspended in assay medium (RPMI 1640 with 10% fetal calf serum) to 10^5 ml^{-1}. The CML assay was performed in round-bottomed microtitre plates. CL at 10^7, 5×10^6, 10^6 and 10^5 per ml were prepared and 0.1 ml samples added per well followed by 0.1 ml of transformed cell (TC) suspension. Plates were centrifuged at 100*g* for 45 s then incubated at 37 °C in 8% CO_2 in air. Each CL:TC ratio was performed in triplicate and controls for spontaneous release of radioactivity included (0.1 ml TC plus 0.1 ml assay medium). Maximum release of label was estimated by incubating 0.1 ml TC with 0.1 ml of 1% Triton X-100. After 4 h, plates were centrifuged again for 10 min to pellet cells and 0.1 ml supernatant collected from each well into small tubes for counting radioactivity released. The percent specific lysis or release was calculated as: (test c.p.m. − spontaneous c.p.m./maximum c.p.m.) × 100. Spontaneous release for all TC was ~12% and for all samples in the table the mean s.d. was 1.3, range 0.1–6.1. LH8 and LH39 are uncloned cell populations whereas LH8.1, 8.2 and 39.1 are cloned cell lines.

and may perhaps be a pseudogene. We have determined part of the DNA sequence which corresponds to the first and second domains of the A and B genes. While the sequence of the B gene is identical to that expected for an *H-2K*^{b} gene, the A gene shows only about 75% homology with this sequence (unpublished results). It is therefore clear that if the A gene does encode an H-2 protein, this protein is no more similar to the *H-2K*^{b} gene product than to any other H-2 molecule[4]. That the B gene is the *H-2K*^{b} gene is shown by a number of functional criteria. First, L cells transformed with the B gene from the H8 cluster express a 45,000 molecular weight polypeptide which carries determinants characteristic of the H-2K^b molecule and which reacts with a number of H-2K^b-specific monoclonals. This suggests that all the epitopes normally associated with the H-2K^b molecule are present on the surface of the transformed L cells. Second, L cells transformed with the B gene are recognized as targets for allogeneic (anti-H-2^b) T_c cell killing and for virus-specific, H-2 restricted T_c-cell killing of infected cells.

These results suggest that a detailed molecular analysis of the determinants recognized by antibodies and the T-cell receptor, using cells transformed with recombinant DNA will be possible. Specifically, L cells transformed with hybrid *H-2* genes, constructed *in vitro*, which contain DNA segments from, say, the *H-2K*^{b} and *H-2D*^{b} genes should help determine whether T_c-cell clones, either allospecific or H-2 restricted, can kill such target cells. In this way, we hope to map the determinants recognized by specific monoclonal antibodies and by the T-cell receptor.

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Formation of transmembrane tubules by spontaneous polymerization of the hydrophilic complement protein C9

Jürg Tschopp, Hans J. Müller-Eberhard & Eckhard R. Podack

Department of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037, USA

The ninth component of complement C9 can undergo circular polymerization in the fluid phase and on lipid membranes. The concomitant hydrophilic–amphiphilic transition is the result of a conformational reorganization of C9 and allows insertion of poly C9 into membranes in the form of a transmembrane protein channel. The ultrastructure of poly C9 resembles that of membrane lesions caused by complement.

FIVE of the more than 20 complement proteins are directly involved in complement-dependent membrane damage and cytolysis[1,2]; these are C5, C6, C7, C8 and C9. Enzymatic cleavage of a single peptide bond in C5 initiates[3] self-assembly of the five proteins into a supramolecular organization termed the membrane attack complex (MAC) of complement[4]. Assembly proceeds from formation of the bimolecular complex C5b–6 to the reaction of C5b–6 with C7, which results in formation of the intermediate complex, C5b–7. After binding of C8 by C5b–7, the C5b–8 complex reacts with multiple C9 molecules, leading to the formation of the MAC. Assembly of the MAC results in the expression of hydrophobicity by the originally hydrophilic proteins. Hydrophobic protein domains seem to be responsible for the insertion of C5b–7 into target membranes[5] and for membrane damage caused by the binding to C5b–7 of C8 and C9 (refs 6, 7). Complement-induced membrane lesions can be visualized by electron microscopy as originally reported by Borsos, Dourmashkin and Humphrey[8]. By negative staining, an individual lesion appears as a circular stain-filled structure, which projects ~12 nm above the membrane surface and which has an inner and outer diameter of 10–11 nm and 21 nm, respectively[9]. As the isolated MAC extracted from complement-lysed cells[10,11] or assembled from the isolated precursor proteins[12–14], has an ultrastructure that resembles the image of an individual membrane lesion, it has been proposed that each membrane lesion actually constitutes one MAC[11,15].

Membrane damage and cytolysis caused by the MAC has been attributed to lipid bilayer perturbation[16,17] and to transmembrane channel formation[15,18,19]. A critical role for C9 in MAC function and structure is indicated by the following: (1) Although it is not an essential component of immune haemolysis, C9 markedly increases the rate of lysis of C5b–8-bearing erythrocytes[20]. (2) Up to six C9 molecules per C8 molecule were reported to be incorporated into the MAC[21] and subsequently, as many as 12–16 molecules per MAC have been estimated[22]. (3) C9 is essential for the formation of the MAC-induced ultrastructural membrane lesions[13,14]. (4) The size of the MAC-produced membrane pore was reported to be dependent on the concentration of C9[23]. (5) C9 within the membrane-bound MAC was found to be the MAC subunit that was predominantly labelled by membrane-restricted-photoactivatable probes[6,7]. (6) Isolated C9 underwent spontaneous polymerization at 37 °C to form circular polymers resembling the MAC in ultrastructure[24].

We report here that C9, a water-soluble plasma protein, can

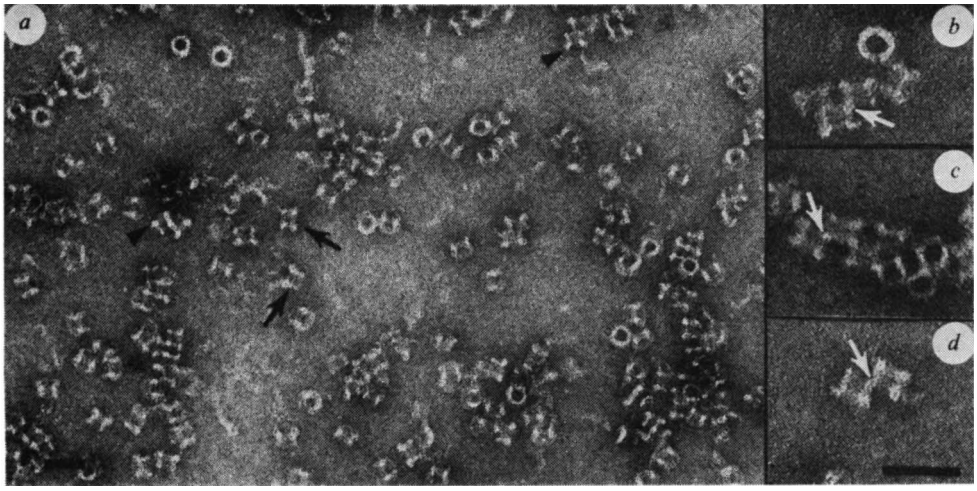

Fig. 1 Ultrastructure of poly C9. C9 was isolated according to Biesecker and Müller-Eberhard[25] and was incubated at a concentration of 5.3 μM in TBS at 37 °C for 64 h. Samples were negatively stained with 2% uranyl formate and examined in a Hitachi model 12A electron microscope at an initial magnification of ×19,550 (*a*). Poly C9 was visualized as a stain-filled tubule with an inner diameter of 11 nm (top views are imaged as rings) and a length of 16 nm (side view, black arrows). The thick-rimmed end comprises a ~3 nm-thick torus with 21 nm outer diameter (black arrowhead). The other end of the tubule terminates in a hydrophobic domain that forms side-by-side aggregates (*b–d*). The white arrows indicate the overlapping areas that presumably represent the hydrophobic segments with a length of 4 nm. Scale bars, 40 nm.

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