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## CONTENTS

### NO. 1, FEBRUARY

A. B. BIJNEN, IEKE SCHREUDER, W. S. VOLKERS, JANNIE PARLEVLIET and J. J. VAN ROOD. The lymphocyte activating influence of the HLA-A region . . . . .	1
V. CECCHI, M. C. BELVEDERE, G. LUCIANI and E. S. CURTONI. A new HLA antigen apparently not controlled by the known HLA loci . . . . .	7
F. GARRIDO, V. SCHIRRMACHER and H. FESTENSTEIN. Studies on H-2 specificities on mouse tumour cells by a new microradioassay . . . . .	15
L. STRZADALA, JADWIGA STEUDEN and C. RADZIKOWSKI. Molecular relationship between H-2 and ML antigens on leukaemia L-1210/v cells . . . . .	29
ELISABETH GOMARD, VÉRONIQUE DUPREZ, YVETTE HENIN and J. P. LEVY. Relationships between H-2 and viral antigens in murine oncornavirus-induced tumours . . . . .	35
R. P. ERICKSON, G. GACHELIN, M. FELLOUS and F. JACOB. Absorption analysis of H-2D and K antigens on spermatozoa . . . . .	47
P. A. EDWARDS, H. W. KUNZ and T. J. GILL III. Genetic studies in inbred rats. VIII. The production of xenoantisera against Ag-B histocompatibility antigens . . . . .	53
M. RIEGER and I. HILGERT. The involvement of a suppressor mechanism in neonatally induced allograft tolerance in mice . . . . .	61
NEW BOOKS . . . . .	69

### NO. 2, APRIL

J. L. ATWELL and J. J. MARCHALONIS. Immunoglobulin gamma chains of a <i>monotreme mammal</i> , the echidna ( <i>Tachyglossus aculeatus</i> ): amino acid composition and partial amino acid sequence . . . . .	73
ANNE-LISE BØRRESEN, K. BERG and T. J. KINDT. A linkage study of rabbit serum high density lipoprotein (HDL) allotypes . . . . .	81
G. INVERNIZZI, G. CARBONE, ANGELA MESCHINI and G. PARMIANI. Multiple foreign non-H-2 determinants on the surface of a chemically-induced murine sarcoma . . . . .	97
U. KOSZINOWSKI and HILDEGUND ERTL. Cytotoxic interactions of virus specific effector cells with virus infected targets of different cell type . . . . .	107
F. GARRIDO, W. SCHMIDT and H. FESTENSTEIN. Immunogenetic studies on Meth-A-vaccinia tumour cells, <i>in vivo</i> and <i>in vitro</i> . . . . .	115
J. W. SHONNARD, B. K. DAVIS, C. T. LADOULIS and T. J. GILL III. The kinetics of IgG and IgM antibody-forming cells in ACI and F344 rats immunized with poly(Glu <sup>52</sup> Lys <sup>33</sup> Tyr <sup>15</sup> ) . . . . .	127
B. K. DAVIS, J. W. SHONNARD and T. J. GILL III. The avidity of IgM antibody in high and low responder rats . . . . .	139

NO. 3. JUNE

ANNE-LISE BØRRESEN. High density lipoprotein (HDL) polymorphisms in rabbit: production of antibody to rabbit allotype (R67) in sheep . . . . .	149
T. SHINOHARA, S. YAMAMOTO and S. ISEKI. Some immunochemical properties of Le <sup>a</sup> - and Le <sup>b</sup> -active substances in human urine . . . . .	159
ELLA M. VAN DEN BERG-LOONEN, BETTY J. DEKKER-SAEYS, S. G. M. MEUWISSEN, L. E. NIJENHUIS and C. P. ENGELFRIET. Histocompatibility antigens and other genetic markers in ankylosing spondylitis and inflammatory bowel diseases . . . . .	167
G. PARMIANI and G. INVERNIZZI. Coincident effect in the graft-versus-host reaction of BALB/c lymphoid cells derived from mice immune either to allogeneic normal tissue or to syngeneic chemically induced fibrosarcomata . . . . .	177
W. DAHR, G. UHLENBRUCK and H. KNOTT. The defect of M <sup>k</sup> erythrocytes as revealed by sodium dodecylsulphate-polyacrylamide gel electrophoresis . . . . .	191
A. AHMED, I. SCHER, A. H. SMITH and K. W. SELL. Studies on non-H-2 linked lymphocyte activating determinants. I. Description of the cell type bearing the MLS product . . . . .	201
SHORT COMMUNICATION	
H. WALTZ and M. ROSE. Crossing over in the HLA system between the SD-1 and SD-3 loci . . . . .	215

NO. 4. AUGUST

EDITORIAL . . . . .	219
R. L. RAISON and J. J. MARCHALONIS. Structural restriction and similarities of the light chains of antibodies to group B streptococcal carbohydrate produced in a single rabbit . . . . .	221
JANE M. MOSELEY, J. J. MARCHALONIS, A. W. HARRIS and J. PYE. Molecular properties of T lymphoma immunoglobulin. I. Serological and general physicochemical properties . . . . .	233
I. F. C. MCKENZIE and MARGARET M. HENNING. The H-2 complex: immunogenicity and enhancement studies of H-2K region alloantigens . . . . .	249
I. F. C. MCKENZIE and MARGARET M. HENNING. The I region transplantation antigens: immunogenicity and enhancement . . . . .	259
C. M. HETHERINGTON and D. P. HUMBER. The effect of pregnancy on lymph node weight in the mouse . . . . .	271
C. C. A. BERNARD, ISABEL M. ROBERTS and I. R. MACKAY. Experimental autoimmune encephalomyelitis in mice: antigen binding lymphocytes to basic protein of myelin in susceptible and resistant strains . . . . .	277
H. C. DUNG, R. L. LAWSON and M. STEVENS. A study of the increased serum level of IgG <sub>1</sub> in 'lethargic' mice combined with a depressed thymus-dependent lymphoid system . . . . .	287

NO. 5, OCTOBER

J. KOISTINEN, R. M. CARDENAS and H. H. FUDENBERG. Anti-IgA antibodies of limited specificity in healthy IgA deficient subjects . . . . .	295
C. HAMERS-CASTERMAN, E. WITTOUCK, G. FLORENT, W. VAN DER LOO, P. DE BAETSELIER and R. HAMERS. Double line phenotypes in rabbit IgG allotypes and their relation to the <i>cis/trans</i> configuration of the IgG markers . . . . .	301
Z. L. HERD and W. E. EDMONDS. Population genetics of Aa and Ab immunoglobulin allotypes in wild rabbits of South-eastern Australia. I. Allotype frequencies . . . . .	315
S. YAMAMOTO. The occurrence of materials cross-reacting with anti-A and -B agglutinins in fruit or seed extracts of higher plants . . . . .	325
ELLA M. VAN DEN BERG-LOONEN, L. E. NIJENHUIS, C. P. ENGELFRIET, T. E. W. FELTKAMP, A. L. VAN ROSSUM and H. J. G. H. OOSTERHUIS. Segregation of HLA haplotypes in 100 families with a myasthenia gravis patient . . . . .	331
M. BOUBELÍK and ALENA LENGEROVÁ. Analysis of genetic differences in early versus late agglutinability with H-2 antibodies of neonatal mouse erythrocytes . . . . .	341
R. P. ERICKSON. Gene expression of a region of chromosome 17 during murine spermatogenesis . . . . .	353

SHORT COMMUNICATIONS

A. M. G. PRETORIUS, D. O. SCHMID, S. CWIK, J. MEYER and E. D. ALBERT. PGM <sub>3</sub> locus and its genetic polymorphism in lymphocytes of the pig . . . . .	363
A. F. GECZY and A. L. DE WECK. Histocompatibility antigens and genetic control of the immune response in guinea-pigs. V. Evidence from further breeding studies for the polygenic control of the cellular immune response to structurally unrelated antigens in the guinea-pig . . . . .	367

NO. 6, DECEMBER

ERNA VAN LOGHEM, MADELEINE BLANC and GERDA DE LANGE. Human IgG3 allotypes, with special reference to a new allotype related to G3m(g) (G3m21) . . . . .	371
ERNA VAN LOGHEM, GERDA DE LANGE, H. MATSUMOTO, T. MIYAZAKI and K. OHKURA. Immunoglobulin haplotypes of two population groups in Iran . . . . .	385
L. RIVAT, J. P. SALIER, M. L. NORTH and F. R. OBERLING. Quantitative studies of Gm allotypes. II. G1m(1), G3m(5) and G3m(21) in sera from healthy caucasoid blood donors, and in a family with the inheritance of a gene responsible for a weak Gm <sup>1, 17; . . . ; 21</sup> haplotype . . . . .	391

O. ŠTARK, E. GÜNTHER, H. W. KUNZ and T. J. GILL III. Comparison of haplotypes of the major histocompatibility complex in the rat. I. The Ag-B7 (H-1 <sup>k</sup> ) and Ag-B8 (H-1 <sup>k</sup> ) haplotypes . . . . .	405
H. W. KUNZ, O. ŠTARK and T. J. GILL III. Comparison of haplotypes of the major histocompatibility complex in the rat. II. Serological analysis of the haplotypes H-1 <sup>a</sup> (Ag-B4), H-1 <sup>d</sup> (Ag-B9) and H-1 <sup>f</sup> (Ag-B10) . . . . .	411
M. J. HOBART, P. J. L. COOK and P. J. LACHMANN. Linkage studies with C6 . . . . .	423
N. R. FARID, R. M. NEWTON, ELKE P. NOEL and W. H. MARSHALL. Gm phenotypes in autoimmune thyroid disease . . . . .	429
AUTHOR INDEX TO VOLUME 4 . . . . .	433

## CYTOTOXIC INTERACTIONS OF VIRUS SPECIFIC EFFECTOR CELLS WITH VIRUS INFECTED TARGETS OF DIFFERENT CELL TYPE\*

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### SUMMARY

The action of Sendai virus specific cytolytic T lymphocytes (CTL) against Sendai virus infected macrophages was found to be H-2 restricted while Sendai virus infected cell lines, including fibroblasts and tumour cells, were lysed across the H-2 barrier to some extent. The properties of the Meth-A tumour cell, which was resistant to lysis by allogenic killer cells was investigated. Sendai virus specific CTL failed to kill Sendai virus infected Meth-A cells but after vaccinia virus infection these target cells were susceptible to lysis by vaccinia virus specific CTL.

### INTRODUCTION

Mice injected with LCM virus, poxviruses or a parainfluenza virus (Sendai) generate virus specific effector lymphocytes (Zinkernagel & Doherty, 1974; Gardner *et al.*, 1975; Koszinowski & Thomssen, 1975; Ertl & Koszinowski, 1976). These effector cells lyse infected target cells *in vitro*. The active cell population in these reactions could be identified as T-cells. It was observed first by Zinkernagel & Doherty (1974) in the LCM virus system that the effector cells, sensitized to a virus, need for a cytolytic interaction with the infected target cells a compatibility of K or D region gene products. This specific property of the virus specific cytolytic T-cells (CTL) drew attention to a possible physiological function of the major histocompatibility complex in cellular immune surveillance against cells bearing neoantigens on the cell surface. For the explanation of the restriction of cell mediated cytolysis to H-2 compatibility two hypotheses have been formulated (Doherty & Zinkernagel, 1975). Firstly the T-cells need for recognition and specific lysis the contact with two antigenic determinants, a self antigen and a viral antigen. The second is, that the virus modifies, in a way unknown at present, the antigenic product of the K or D region, this new antigen is recognized by the T-cells. Recent findings in chimeric mice suggest

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Abbreviations: CTL, cytolytic T lymphocytes; CMC, cell mediated cytolysis; EID<sub>50</sub>, 50% egg infective dose; LCM, lymphocytic choriomeningitis; LD<sub>50</sub>, 50% lethal dose; MEM, minimal essential medium; T-cell, Thymus derived cell.

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(Pfizenmaier *et al.*, 1976), that the second theory, the 'altered self' concept seems to be more likely, since modifications of allogenic cells can also be recognized, provided the T-cell is tolerant to these antigens.

## MATERIALS AND METHODS

### *Viruses*

*Sendai virus*. Strain D-52 (Behringwerke AG, Marburg/Lahn) was propagated in the allantoic fluid of 10-day-old fertile eggs. The twenty-seventh passage was used throughout. The virus was diluted to a titre of  $10^7$  EID<sub>50</sub>/ml.

*Vaccinia virus*. Strain WR was propagated in Vero (African green monkey kidney) or in OMK (owl monkey kidney) cells and diluted to a titre of  $10^{5.5}$  TCID<sub>50</sub>/ml.

*LCM virus*. Strain WE-3 kindly provided by Dr Lehmann-Grube, Hamburg, was propagated in L-929 cells and used in a concentration of 300 LD<sub>50</sub>/ml.

### *Mice*

Inbred mice of strains C3H (H-2<sup>k</sup>), DBA-2 (H-2<sup>d</sup>) and C57B1/6 (H-2<sup>b</sup>) purchased from B. L. Bomholdgard, Ry, Denmark, were used at the age of 8–12 weeks.

### *Indirect immunofluorescence*

Indirect immunofluorescence of viral surface antigens on viable infected cells was performed as described earlier (Koszinowski & Ertl, 1975).

### *Target cells*

L-929 cells (H-2<sup>k</sup>) were grown as monolayers in Eagle's minimal essential medium supplemented with 10% calf serum and 100 µg/ml penicillin and streptomycin. Mastocytoma P-815 (H-2<sup>d</sup>), Meth-A (H-2<sup>d</sup>), SL-2 (H-2<sup>d</sup>) and EL-4 (H-2<sup>b</sup>) tumour cells, kindly provided by Dr Festenstein, London, were passaged in the peritoneal cavity of syngeneic mice. These tumour lines were maintained *in vitro* as suspension cultures in Eagle's minimal essential medium supplemented with 10% fetal calf serum, 200 mM glutamine, 1% non-essential amino acids and 100 µg/ml penicillin and streptomycin. Macrophages were harvested 3 days after i.p. injection of 2 ml thioglycolate and separated from other cells by affinity to glass or plastic surfaces and trypsin resistance.

### *Infection of target cells*

A volume of 5 ml virus suspension (about 5 infective units/cell) was added to  $1 \times 10^6$  L-929 monolayer cells. One hour later the medium containing virus was discarded and fresh medium was added. Tumour cells,  $1-5 \times 10^6$ , were suspended in medium containing virus for 1 hr, centrifuged and then resuspended in fresh medium. Macrophages were cultivated for 4 hr in petri dishes. The non-adherent cells were removed and virus was added. After 1 hr incubation the virus containing fluid was replaced by fresh medium. Efficiency of infection was monitored 10 hr later by indirect immunofluorescence.

### *Sensitization to alloantigens*

Mitomycin C treated stimulator cells,  $1 \times 10^6$ , were incubated with  $4 \times 10^6$  responder spleen cells in 2 ml Dulbecco's modified medium supplemented with 5% FCS and 100 µg/ml penicillin and streptomycin in a CO<sub>2</sub> atmosphere at 37°C. As stimulator cells

spleen cells or tumour cells from different inbred strains were used. The cells were harvested 5 or 6 days later and their activity on the appropriate  $^{51}\text{Cr}$ -labelled target cells was tested in a 6–10 hr assay. Ratio of attacker to target cells ranged between 20:1 and 50:1.

#### *Cytotoxic assay*

The assay was performed with some modifications according to the method of Wagner (1973). The amount of  $1-5 \times 10^4$ , about 6–14 hr infected or not infected  $^{51}\text{Cr}$ -labelled target cells, were incubated with  $1-5 \times 10^6$  lymphocytes from normal mice or mice sensitized to virus 6 days previously, in a volume of 1.0 ml in  $1 \times 5$  cm round bottom plastic tubes. Spontaneous lysis was determined by adding medium instead of lymphocytes, the maximal lysis was determined by adding 1 M HCl to the target cells. After 8–18 hr incubation supernatant and cells were harvested separately. The percentage  $^{51}\text{Cr}$ -release was calculated using the formula:

$$\text{Percentage } ^{51}\text{Cr}\text{-release} = \frac{\text{counts in the supernatant}}{\text{counts in the supernatant} + \text{in the pellet}} \times 100.$$

Specific lysis was calculated by subtraction of percentage  $^{51}\text{Cr}$ -release in presence of normal lymphocytes from percentage  $^{51}\text{Cr}$ -release in the presence of immune lymphocytes. Assays were done at least in triplicate, s.d. values of the data presented here were less than 5%. Significance was calculated using the Student's *t*-test.

#### *Cold cell inhibition assay*

The assay was performed according to the method of Herbermann *et al.* (1976). Inhibition was calculated as follows:

$$\text{Percentage inhibition of specific lysis} = \frac{\% \text{ specific lysis in immune control} - \% \text{ specific lysis in presence of inhibitory cells}}{\% \text{ specific lysis in immune control}} \times 100.$$

Inhibitory cells were added in different ratios varying from 2:1 to 50:1 cold inhibitory cells to labelled target cells.

## RESULTS

#### *Virus specificity of cell mediated cytotoxicity*

Mice from the inbred strain C3H were injected intraperitoneally with either  $10^{5.5}$  TCID<sub>50</sub> vaccinia virus,  $10^7$  EID<sub>50</sub> Sendai virus or 300 LD<sub>50</sub> LCM virus. Spleen cells were harvested 7 days later. Cytolytic activity was tested on macrophage targets infected with the sensitizing viruses. Lymphocyte activity was restricted to the virus according to which the lymphocyte donors were sensitized to (Table 1). Cross-reactivity could not be observed. Restriction of activity to the sensitizing virus reflects the antigenic specificity of the new determinant induced by the viral infection on the cell surface.

#### *H-2 gene restricted activity of CL on infected macrophages*

Sendai virus specific sensitized cells from C3H (H-2<sup>k</sup>), DBA/2 (H-2<sup>d</sup>) and C57Bl/6 (H-2<sup>b</sup>) mice were incubated with normal and Sendai virus infected macrophages bearing each



TABLE 1. Virus specificity of T-cell mediated cytotoxicity

Target cells	Percentage specific cytotoxicity*		
	Vaccinia sensitized C3H lymphocytes	Sendai sensitized C3H lymphocytes	LCM sensitized C3H lymphocytes
Normal macrophages	0.2 ± 0.3	0.7 ± 0.5	-3.3 ± 0.3
Macrophages, infected with vaccinia virus	36.9 ± 0.2	7.8 ± 0.9	5.3 ± 0.5
Macrophages, infected with Sendai virus	1.1 ± 0.6	42.2 ± 1.4	1.7 ± 0.1
Macrophages, infected with LCM virus	-0.4 ± 0.7	-1.5 ± 0.1	27.4 ± 0.7

\* The activity of C3H spleen cells of mice sensitized 6 days previously was tested in a 8 hr <sup>51</sup>Cr-release assay on target cells at a ratio effector cells : target cells = 100 : 1.

of the three haplotypes. The effector cells killed only syngeneic infected target cells (Table 2).

#### *Lysis of allogenic infected target cells*

The same sensitizing procedure was performed as in the preceding experiment. Sendai virus infected L-929 as well as infected tumour cells (P-815, Meth-A, EL-4) were used as targets in this experiment. In contrast to the results found with macrophage targets, cross killing of allogenic H-2 incompatible target cells could be observed.

TABLE 2. H-2 dependency of T-cell mediated cytotoxicity. Differences between primary cells and permanent cell lines

Target cells	Percentage specific lysis from target cells infected with Sendai virus		
	Effector cells		
	C3H	DBA/2	C57B1/6
C3H macrophages	18.8	-1.2	0.8
L-929	30.8	11.6	9.9
DBA/2 macrophages	-6.8	20.5	2.6
P-815	6.9	16.9	1.7
C57B1/6 macrophages	0.1	0.4	12.6
EL-4	-1.1	18.0	25.0

Ratio of attacker cells to target cells 50 : 1. Incubation time 12 hr. Data represent the mean of four wells per group. Differences are statistically significant ( $P < 0.01$ ).

*Cytolytic interactions with Meth-A target cells*

In experiments with Sendai virus infected Meth-A cells this target was not killed by the effector cells. Virus specific immunofluorescence showed that this cell could be infected with Sendai virus. We therefore tested to see if this target cell was resistant to lysis by allogenic killer cells. Allogenic lymphocytes were sensitized *in vitro* to H-2<sup>d</sup> by incubation with mitomycin C treated DBA/2 cells. These effector cells were unable to lyse Meth-A cells, despite the fact that they lysed the H-2<sup>d</sup> tumours P-815 and SL-2 (Table 3) very well. In a second approach we tested the possibility of sensitizing lymphocytes directly to Meth-A cells *in vitro*. Cultures were set up with P-815, SL-2 and Meth-A as mitomycin C treated stimulator cells and C3H (H-2<sup>k</sup>) lymphocytes as responder cells. After 5 days there was a specific cytolytic response to P-815 and SL-2 but no response at all to Meth-A (Table 3). Treatment of stimulator Meth-A with *Vibrio cholerae* neuraminidase had no increasing effect.

Since Meth-A expresses H-2<sup>d</sup> SD antigens we tested to see if these cells could inhibit the cytolytic interaction between anti H-2<sup>d</sup> killer cells and P-815 as targets. There was a clear dose-response relationship between the numbers of inhibitor cells in the test and inhibition, when tumour cells, bearing H-2<sup>d</sup> were used as cold inhibitory cells. The slope of the inhibition curve after adding Meth-A cells was similar to that produced by the positive P-815 inhibition control. This shows that the antigenic determinants recognized on P-815 cells by anti H-2<sup>d</sup> specific CTL are present on the Meth-A tumour cells as well (Fig. 1). The allogenic control cell EL-4 did not inhibit the cytolytic interaction.

TABLE 3. Behaviour of Meth-A tumour cells as stimulator and target cells

Sensitization	Effector phase (target cells)	Percentage specific lysis
H-2 <sup>k</sup> → H-2 <sup>d</sup> lymphocytes	P-815	61.7
	SL-2	58.3
	Meth-A	0.8
H-2 <sup>k</sup> → P-815	P-815	65.8
	SL-2	59.7
H-2 <sup>k</sup> → SL-2	P-815	63.3
	SL-2	62.5
H-2 <sup>k</sup> → Meth-A	P-815	1.2
	SL-2	0.8
	Meth-A	0.4
H-2 <sup>d</sup> → Sendai virus	P-815 Sendai virus infected	30.2
	Meth-A Sendai virus infected	2.4
H-2 <sup>d</sup> → Vaccinia virus	P-815 Vaccinia virus infected	28.6
	Meth-A Sendai virus infected	26.5

Allogenic lysis: ratio of effector cells to target cells 20:1; incubation time 6 hr.

Virus specific lysis: ratio of effector cells to target cells 50:1; incubation time 12 hr.

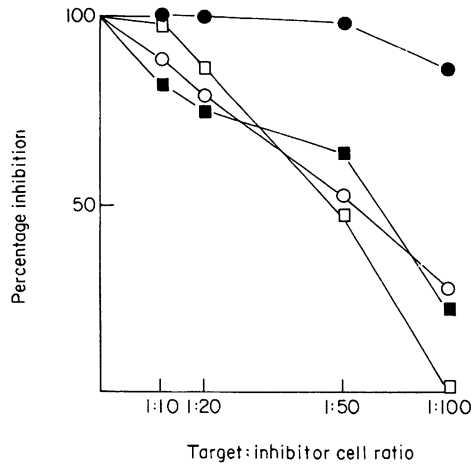


FIG. 1. Percentage inhibition of cytolytic activity of C3H anti H-2<sup>d</sup> killer cells towards  $1 \times 10^4$  <sup>51</sup>Cr-labelled P-815 cells obtained with different numbers of cold EL-4 (●—●), P-815 (□—□), SL-2 (○—○) or Meth-A (■—■) tumour cells.

Ratio of attacker: <sup>51</sup>Cr-labelled target cells 20:1. Incubation time 8 h: control cytolysis without inhibitory cells added was 48%.

However, Meth-A cells were not always resistant to cell mediated cytolysis. When we infected this cell with vaccinia virus it could be very well lysed by syngeneic effector cells sensitized to vaccinia virus (Table 3).

## DISCUSSION

Cell mediated specific lysis of targets modified by viral infections (Zinkernagel & Doherty, 1974; Gardner *et al.*, 1975; Koszinowski & Thomssen, 1975; Ertl & Koszinowski, 1976), tumour antigens (Schradler & Edelman, 1976), chemical agents (Shearer, 1974), the male histocompatibility antigen of the mouse (Gordon *et al.*, 1975) and minor histocompatibility antigens (Bevan, 1975) is restricted to K or D compatibility between target and effector cell. Data presented here show the same restrictions in the Sendai virus infection when primary cells were used as targets.

Using virus infected tumour cell lines as target cells different patterns of reactivity were observed. To some degree there was killing of non-infected tumour target cells. Infected target cells were specifically lysed across the H-2 barrier. One of the target cells could be lysed after infection with vaccinia but not with Sendai virus.

Cross-reactivities could be due to different mechanisms. Firstly, some non-specific lysis may be caused by higher spontaneous lysis of tumour cells infected with virus. Very active CTL can lyse target cells bearing modifications they are not sensitized to (Starzinski-Powitz *et al.*, 1976). The activity of killer cells without specificity has to be excluded. The fact that the reactivity across the H-2 barrier was specific for the 'modifying' virus argues for an effector cell specificity since cells infected with unrelated viruses were not lysed (unpublished results).

Similar cross-reactivities were seen when TNP modified tumour cell were used as targets for TNP specific CTL (Shearer *et al.*, 1975). Sendai virus specific CTL induced in DBA/2

(H-2<sup>d</sup>) mice killed infected EL-4 targets. The same activities were obtained in cross killing experiments with CTL from mice sensitized to H-2<sup>d</sup> tumours (Schrader & Edelman, 1976). This means that CTL recognize antigenic determinants on tumours, which are not identical to the modified antigenic products of K or D region these cells should normally express. Possible explanations are activation of endogenous viruses, recognition of derepressed antigens (Bodmer, 1972) or of antigens not identical with private H-2 specificities (Nabholz *et al.*, 1974). Cross-reactivities against similar H-2 structures (Hennig *et al.*, 1975) seem unlikely since virus specific CTL can even discriminate 'point' mutations in the K region (Doherty *et al.*, 1976).

The observation that Meth-A cells neither sensitize allogenic cells *in vitro* nor are they susceptible to cytolysis by allogenic killer cells seems to indicate deficiencies of antigenic expressions of the H-2 complex on these cells.

Since these antigens can be demonstrated by serological means (Garrido *et al.*, 1976b) one could argue that these antigens are not accessible to the T-cell receptors. But positive recognition was found in the cold cell inhibition experiments. This suggests an inhibition of the lethal hit (Golstein & Smith, 1976). After vaccinia virus infection, virus specific CMC is possible. Similar results were found by Matossian-Rogers and coworkers (personal communication). This is not due to cytopathic effects of the virus itself as no lysis was obtained after Sendai infection. Therefore activities of vaccinia virus on membrane properties by rearrangement of surface structures or by removal of components interfering with T-cell mediated lysis could explain these data.

In most of these experiments fibroblasts or macrophages served as targets for virus specific lysis. It has recently been shown, that tumour cells show immunological cross-reactivities with alloantigens (Invernizzi & Parmiani, 1975; Bowen & Baldwin, 1975). By serological means expressions of new antigens on tumour cells have been found (Pellegrino *et al.*, 1976; Garrido *et al.*, 1976a, b). To summarize: in this paper we present preliminary data about virus specific reactions against infected tumour cells. When tumour cells were used as targets cross-reactivities with allogenic infected target cells could be observed. A tumour target cell of H-2<sup>d</sup> haplotype resistant to lysis by allogenic anti H-2<sup>d</sup> killer cells, but as susceptible as a vaccinia virus infected target cell to lysis by virus sensitized effector cells is described.

#### ACKNOWLEDGMENTS

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