A NEW SENSITIVE ASSAY FOR ANTIBODY AGAINST CELL SURFACE ANTIGENS BASED ON INHIBITION OF CELL-DEPENDENT ANTIBODY-MEDIATED CYTOTOXICITY

I. Specificity and Sensitivity*

BY PHIL HALLORAN, VOLKER SCHIRRMACHER, AND HILLIARD FESTENSTEIN

(From the Tissue Immunology Research Unit, The London Hospital Medical College, University of London, London, England)

When antibody binds to target cell antigens alterations occur in the Fc portion of the immunoglobulin molecule which can lead to activation of various immunological effector mechanisms, e.g. the complement (C) system or cytotoxic effector cells. The activation of such effector mechanisms can be used for assaying antibody activity. C-dependent antibody assays have been widely used but often suffer from low reproducibility and may give different results with different sources of C.

Cell-dependent antibody-mediated cytotoxicity (1, 2) has proven to be a powerful assay for antibody in some systems (3). However, the interaction of lymphoid cells with antibody-coated target cells does not always result in target cell lysis. For example, human peripheral blood leukocytes lyse alloantibody-coated human lymphocytes with great sensitivity (4, 5). In contrast, mouse spleen cells, which are a good source of effector cells[1] in some xenogeneic systems (6–9) do not lyse alloantibody-coated mouse lymphocytes (P. Halloran, unpublished data; I. C. M. Maclennan and I. MacKenzie, personal communications). Reasons for these striking species differences are at present unknown, but these findings imply that the induction of target cell lysis in cell-dependent antibody-mediated systems cannot be generally used to detect antitarget cell antibody.

In the present series we describe a new widely applicable assay for antibody which is based on inhibition rather than induction of cell-dependent antibody-mediated cytotoxicity. The cytotoxicity test system consists of (*a*) normal mouse

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[1] The effector cells probably represent only a small fraction of the total spleen cell population. For the nature of the cells, see Discussion.

spleen cells (effector cells), (*b*) ^{51}Cr-labeled chicken erythrocytes (CRBC)[2] (target cells), and (*c*) minute amounts of rabbit anti-CRBC antibody (RACA) (8). In a preliminary communication (10) we showed that anti-*H-2* sera directed against alloantigens in the effector cell population can specifically inhibit the cytotoxicity in this test system. It will be shown in this series that a variety of allogeneic and xenogeneic antisera capable of reacting with surface antigens in the effector cell population lead to inhibition of target cell lysis in the above system. This reaction forms the basis of the cytotoxicity inhibition assay (CIA) which will be presented as a new antibody assay, independent of C. The specificity and sensitivity of the CIA will be described in this report. The advantages of this assay over conventional assays and some possible applications of it will be discussed.

Materials and Methods

Materials. Culture medium: Eagles MEM (The Wellcome Research Laboratories, Kent, England) with 0.013 M bicarbonate and antibiotics (Gentamycin 5 μg/ml, cloxacillin 125 μg/ml, and ampicillin 125 μg/ml) and 5% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.). Culture tubes: flat-bottomed 9 × 44 mm polystyrene tubes (The Metal Box Co., Surrey, England). Isotope: radioactive sodium chromate (^{51}Cr, 1 mCi/ml) (The Radiochemical Centre, Amersham, England).

Animals. Young adult male and female mice were used. Strains CBA/H, BALB/c, DBA/2, C57Bl/6, C57Bl/10ScSn (abbreviation "B10"), B10.A, B10.D2, B10.BR, and nude (nu/nu) mice were bred in this laboratory or obtained from the Laboratory Animal Centre, Carshalton, Surrey, England. Strains B10.A(2R), B10.A(4R), B10A(5R), B10.HTT, A.TH, B10.D2(M504), and B10.AKM were bred in this center from breeding pairs obtained from Dr. Peter Demant, Institute of Experimental Biology and Genetics, Prague, Czechoslovakia; from Dr. Donald Shreffler, University of Michigan, Ann Arbor, Mich.; and from Dr. H. O. McDevitt and B. Deak, Stanford University, Stanford, Calif. The congenic and recombinant strains used were discovered and established by Snell, Shreffler, Demant, Stimpfling, Gorer, Ivanyi, and Egorov.

Antisera

ANTI-*H-2* ALLOANTISERA. Table I contains the details of the anti-*H-2* sera used. Alloantisera 1–6 were raised by injecting at weekly intervals 10^7 spleen cells (or P815 tumor cells for serum 5) intraperitoneally into appropriate recipients. Sera obtained after 2–3 mo were heat inactivated and stored at —20°C. Sera 7–13 were obtained from the Transplantation and Immunology Branch, NIAID, NIH, Bethesda, Md. Details of the raising and testing of these sera are contained in the NIH catalogue[3] and in its 1973 supplement.

RABBIT ANTIMOUSE GAMMA GLOBULIN (R ANTI-MGG). Rabbits were immunized at weekly intervals with a suspension of *Proteus* OX2 organisms which had been coated with mouse anti-*Proteus* antibodies and carefully washed. Anti-MGG antibodies were immunosorbent purified using MGG-linked

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[2] *Abbreviations used in this paper:* CDL, C-dependent lysis; CIA, cytotoxicity inhibition assay; CRBC, chicken erythrocytes; Ia antigens, I-region-associated antigens; NMG, normal mouse gamma globulin; NRG, normal rabbit gamma globulin; RACA, rabbit anti-CRBC antibody; R anti-BAθ, rabbit antimouse brain-associated-θ; R anti-MBLA, rabbit antimouse bone marrow-derived lymphocyte antigen; R anti-MGG, rabbit antimouse gamma globulin (from immune serum).

[3] Snell, G. D. 1968. Catalogue of Mouse Alloantisera. Transplantation Immunology Branch Publication, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Md.

TABLE I
H-2 Antisera

No.	Serum designation	Recipient-donor strains	Recipient-donor genotypes	Anti-*H-2* specificities
1	"k anti-d"	CBA/H anti-BALB/c	$H\text{-}2^k$ anti-$H\text{-}2^d$	multiple
2	"d anti-k"	BALB/c anti-CBA/H	$H\text{-}2^d$ anti-$H\text{-}2^k$	multiple
3	"k × d anti-b"	(CBA/H × BALB/c)F_1 anti-C57BL/6	$(H\text{-}2^k/H\text{-}2^d)$ anti-$H\text{-}2^b$	multiple
4	"b anti-d"	C57BL/6 anti-BALB/c	$H\text{-}2^b$ anti-$H\text{-}2^d$	multiple
5	"k anti-P815Y"	CBA/H anti-P815Y	$H\text{-}2^k$ anti-$H\text{-}2^d$	multiple
6	"b anti-5R"	B10 anti-B10.A(5R)	$H\text{-}2^b$ anti-$H\text{-}2^{i5}$	multiple
7	"D-4"	(B10.AKM × 129)F_1 anti-BIO.A;	$(H\text{-}2^m/H\text{-}2^{bc})$ anti-$H\text{-}2^a$	4
8	"D-8"	(B10 × A.SW)F_1 anti-BIO.M;	$(H\text{-}2^b/H\text{-}2^s)$ anti-$H\text{-}2^f$	8, 9, 37
9	"D-23"	(B10 × LP.R III)F_1 anti-B10.A(2R);	$(H\text{-}2^b/H\text{-}2^r)$ anti-$H\text{-}2^{h2}$	23
10	"D-25"	(B10.D2 × C3H.NB)F_1 anti-B10 R.III;	$(H\text{-}2^d/H\text{-}2^p)$ anti-$H\text{-}2^r$	25, 11, 18, 54
11	"D-31"	(B10 × A)F_1 anti-B10.D2;	$(H\text{-}2^b/H\text{-}2^a)$ anti-$H\text{-}2^d$	31, 34
12	"D-33"	(B10.D2 × A)F_1 anti-B10.A(5R);	$(H\text{-}2^d/H\text{-}2^a)$ anti-$H\text{-}2^{i5}$	33, 53, 54
13	"D-35"	B10.A (2R) × A.CA)F_1 anti-B10.Y.	$(H\text{-}2^{h2}/H\text{-}2^f)$ anti-$H\text{-}2^{pa}$	35, 34, 41

Sepharose 4B beads, which were prepared as described (11). Absorbed antibodies were re-eluted by suspending the beads for 15 min at 37°C in 0.1 M acetic acid, pH 3.0. The neutralized supernates had a specific agglutination titer against MGG-coated sheep erythrocytes (SRBC) (12) of more than $1{:}10^6$.

RABBIT ANTIMOUSE BRAIN-ASSOCIATED-θ (R ANTI-BAθ). Rabbits were immunized twice with mouse brain tissue emulsified in complete Freund's adjuvant (13). Antisera obtained 2–3 mo after immunization were inactivated for 30 min at 56°C before they were immunosorbent purified on mouse brain tissue. 5-ml antiserum was incubated for 1 h on ice with brain tissue from 15 BALB/c mice. Absorbed antibodies were re-eluted from the washed tissue by suspending it for 15 min at 37°C in 5 ml 0.1 M acetic acid, pH 3.0. The neutralized supernate was further absorbed with MGG-Sepharose beads and with spleen cells from nude mice. It was then dialyzed against MEM.

AKR/J ANTI-θC3H SERUM (ANTI-THY 1.2). AKR/J anti-θC3H serum pretested for specificity (14), was kindly donated by Dr. R. F. Kerbel, Queen's University, Kingston, Canada.

RABBIT ANTIMOUSE BONE MARROW-DERIVED LYMPHOCYTE ANTIGEN (R ANTI-MBLA). Rabbits were immunized twice with spleen cells from adult thymectomized, 850 R irradiated, and bone marrow-reconstituted CBA/H mice (15). The IgG fractions of antisera obtained after 2–3 mo were prepared by DEAE-cellulose chromatography (16). They were absorbed with MGG-Sepharose beads, mouse brain tissue, and CBA/H thymus cells.

RACA. Rabbits were immunized at weekly intervals with about 10^9 washed fresh CRBC and bled after 5–6 wk. The heat-inactivated serum had a titer in the antibody-dependent cell-mediated cytotoxicity assay (see below) of more than $1{:}10^6$.

All xenogeneic antisera were found to be specific in conventional assays (C-dependent lysis [CDL], passive hemagglutination, and indirect immunofluorescence) (Volker Schirrmacher, unpublished observations).

C-Dependent Microcytotoxicity. Our standard method for CDL was a microassay. The test, which has been described previously (17) was performed in Microtest 3034 plates (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). 1 μl of cell suspension (10^6/ml) and 1 μl of antiserum were in-

cubated together at room temperature, for 30 min. Then 5 μl of diluted rabbit or guinea pig C was added and the mixture was incubated at room temperature for 30 min. The results were read directly under phase contrast.

Indirect Immunofluorescence. Cells were labeled with rabbit antisera in excess for 1 h on ice, washed, and then incubated again for 1 h on ice with 1:4 diluted fluorescein-labeled goat antirabbit gamma globulin. The washed cells were observed for membrane fluorescence under a fluorescence microscope using a BG 12 (1 mm) filter.

CIA. To culture tubes were added: 1 or 2 $\times$ 10^4 fresh CRBC labeled with ^{51}Cr (18), 1–4 $\times$ 10^6 twice washed normal mouse spleen cells, and RACA to give a final concentration of 1/25,000–1/100,000. Inert SRBC (10^7/ml) were added to reduce the spontaneous ^{51}Cr release (6). The serum to be tested was either added directly to the final incubation mixture or preincubated with "third-party" cells carrying the appropriate determinants, which were then washed and added to the incubation mixture.

The exact composition of the final incubation mixture and the duration of incubation varied according to the specific requirements: method A (to achieve a convenient short-term assay), 3- to 4-h incubation; effector to target cell ratio, 100–200:1; RACA concentration, 1/25,000; and method B (to achieve maximum sensitivity), 18-h incubation; effector to target cell ratio, 50:1; RACA concentration, 1/100,000. The basis for the selection of these quantities is given in the second paper of this series. Where economy in consumption of antiserum was required, a final volume of 250 μl was employed; otherwise, the final volume was 1,000 μl.

Incubations were performed in a desiccator at 37°C in a 10% O_2 and 5% CO_2 atmosphere. After incubation (3, 4, or 18 h) the tubes were centrifuged and aliquots of the supernates were removed. The residual incubation mixtures and the supernatant aliquots were counted separately in a Packard Autogamma well-type scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.), and the percent ^{51}Cr release was calculated. The means and standard deviations from the results of triplicate tubes were obtained, and differences were assessed for significance by a Student's *t* test. The inhibition of ^{51}Cr release by the test antiserum was also tested against autologous, parent, or *H-2* identical cells. Inhibition of cytotoxicity was considered specific only when greater than in these controls.

The CIA results could be expressed in several ways: (*a*) percent ^{51}Cr release; (*b*) reduction in percent ^{51}Cr release, i.e. percent ^{51}Cr release in presence of normal serum minus percent ^{51}Cr release in presence of antiserum (any nonspecific reduction observed with autologous cells was corrected for by subtraction); and (*c*) percent inhibition of cytotoxicity, i.e., the reduction in percent ^{51}Cr release expressed as a percentage. Examples of data expressed in each of these ways are included in the Results.

Results

Specificity of the CIA

ANTI-*H-2*. When normal mouse spleen cells (effector cells) were incubated with antibody-coated and ^{51}Cr-labeled CRBC (target cells), the spleen cells were induced to lyse the CRBC as demonstrated by the ^{51}Cr released into the supernate. The addition of anti-*H-2* sera to the incubation mixture containing spleen cells bearing the appropriate *H-2* antigens led to inhibition of the cytotoxicity. Fig. 1 illustrates the specificity of this reaction, comparing the effect of antiserum with that of normal mouse serum. Serum "d $\times$ k anti-b" (anti $H\text{-}2^b$) strongly inhibited the cytotoxicity of B10 ($H\text{-}2^b$) spleen cells; yet it had no specific effect on the cytotoxicity of BALB/c ($H\text{-}2^d$) or CBA/H ($H\text{-}2^k$) spleen cells. Similarly, the sera "b anti-d" (anti-$H\text{-}2^d$) and "k anti-d" (anti-$H\text{-}2^d$) gave strong inhibitions of ^{51}Cr release by BALB/c spleen cells, and serum "d anti-k" (anti-$H\text{-}2^k$) inhibited the ^{51}Cr release by CBA/H spleen cells. As predictable on the

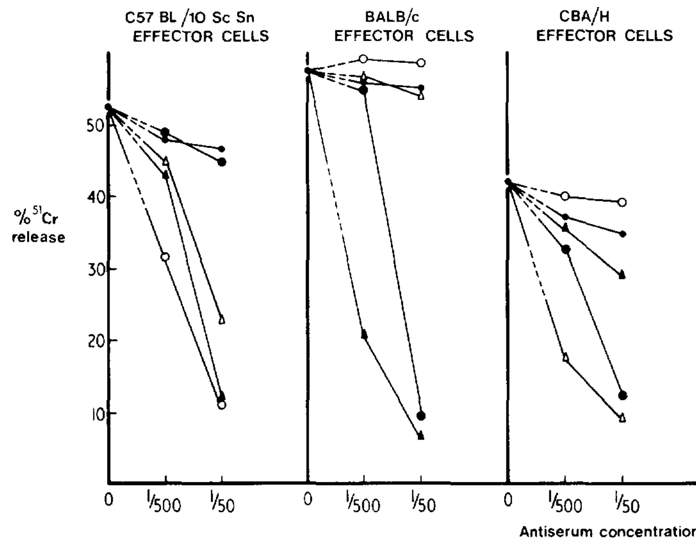

FIG. 1. Specificity of the CIA. The cytotoxicity of spleen cells from three different strains of mice (C57Bl/10 Sc Sn (*H-2^b^*), BALB/c (*H-2^d^*), and CBA/H (*H-2^k^*) was determined in absence or presence of either normal mouse serum (control) or various anti-*H-2* sera. For test conditions see method A. The final dilutions of the sera are indicated on the abscissa. (•—•) normal mouse serum; (O—O) "k x d-anti b" (anti-*H-2^b^*); (●—●) "b anti-d" (anti-*H-2^d^*); (▲—▲) "k anti-d" (anti-*H-2^d^*); and (Δ—Δ) "d anti-k" (*anti*-H-2^k^). Background ^{51}Cr release without RACA was 2%.

basis of public specificities in the *H-2* chart (19), inhibition of cytotoxicity was observed with serum "b anti-d" tested against CBA/H and with the sera "k anti-d" and "d anti-k" tested against B10 spleen cells. None of the sera inhibited the cytotoxicity of cells which were autologous, parent, or *H-2* identical to the strain in which the antibody was raised. Thus, the ability of anti-*H-2* sera to inhibit cytotoxicity in our system was specific. The pattern of inhibition described above suggested that the activity of the sera was directed against "serologically-defined" private and public specificities of the *H-2* complex.

For more definitive elucidation of the regions in the *H-2* complex controlling the antigens recognized by the CIA, we investigated the inhibitory effects of well-characterized NIH *H-2* antisera[3] against cells from various inbred, congenic, and recombinant strains. Table II contains the results of these studies, comparing the antigens expected to be present on the cells (on the basis of their *H-2* genotype) with the results obtained in the CIA. A complete correlation was found between the pattern of antigens expected and the pattern of inhibition observed. The sera used were directed against private specificities located in the *H-2* K region (*H-2K.23*, *H-2K.31*, and *H-2K.33*) or *H-2* D region (*H-2D.4*) and against a public specificity mapping in the *H-2* K region (*H-2K.8*). Our results therefore show that the CIA can detect private and public specificities of the *H-2* K and *H-2* D genes.

Furthermore, other serological specificities located in the *H-2* complex could be detected in the CIA as well. Between the *H-2K* and the *H-2D* loci, there is a

TABLE II

Results of Testing anti-H-2 Sera of Narrow Specificity in the CIA

Effector cells	*H-2* type	Predicted reactions‡					Inhibition of cytotoxicity§ in presence of antiserum				
		D-4	D-8	D-23	D-31	D-33	D-4	D-8	D-23	D-31	D-33
							%	%	%	%	%
DBA/2	d	+	+	−	+	−	41**	26**	0	51***	0
CBA/H	k	−	+	+	−	−	2	20**	33**	3	8
B10	b	−	−	−	−	+	10	10	15	10	85***
							0	3	8	3	58***
B10.D2	d	+	+	−	+	−	64***	57***	0	71***	7
B10.D2 (M504)	d	+	+	−	+	−	43***	53***	10	77***	13
B10.HTT	t3	+	−	−	−	−	50***	3	9	6	NT
B10.AKM	m	−	+	+	−	−	7	69***	66***	10	7
							8	32***	37***	5	NT
B10.BR	k	−	+	+	−	−	12	47**	47**	6	0
B10.A	a	+	+	+	−	−	55***	45***	65***	−5	−8
B10.A(2R)	h2	−	+	+	−	−	4	38***	50***	0	−4
B10.A (5R)	i5	+	−	−	−	+	32***	14	11	11	79***
(B10.A(2R) × B10.AKM)F_1	(h2 × m)F_1	−	+	+	−	−	7	64***	57***	14	NT
(B10.A(2R) × B10.D2)F_1	(h2 × d)F_1	+	+	+	+	−	41***	59***	41***	63***	−7

‡ Predictions are based on the *H-2* genotype of the mouse strain and on the specificities of the sera determined by complement-dependent lysis[2]. The principal *H-2* specificity is indicated by the number of the antiserum, i.e., D-8 is directed at specificity *H-2K.8*. Other specificities are present in some of these sera (see Table I) but are not a consideration in the strains selected here. Ia specificities may be present in some of these sera (e.g., D-33 contains anti Ia.9-see text) but cannot account for most of the reactions presented in this table.

§ i.e., (% ^{51}Cr release with normal serum − % ^{51}Cr release with test serum)/(% ^{51}Cr release with normal serum) × 100; the numbers indicate arithmetic means; data based on five experiments, using the 4-h test (method A); cells from B10 and B10.AKM were tested twice and the data are presented to show the reproducibility. Asterisks indicate the level of significance in a Student's t test. *0.05 > P > 0.01; **0.01 > P > 0.001; and ***0.001 > P.

series of genes which control antigens with a restricted tissue distribution. These antigens were named Ia antigens (I-region-associated antigens) because the region in which they are localized is called the immune response region (20). The results presented in Table III indicate that these specificities can be detected in the CIA. The sera "b anti-5R" and "D-33" (both anti-H-2^{i5}) were tested against strains selected to differ at various *H-2* regions, particularly I and S. "b anti-5R" specifically inhibited the cytotoxicity of B10.A(5R), B10.A, B10.A(2R), and B10.HTT but not of B10, B10.A(4R), and A.TH. Since B10.A(2R) and B10 A(4R) are identical except at I-C and S, the inhibition of the cytotoxicity of B10.A(2R) is explainable only on the basis of antigens mapping in these regions. A similar argument holds true for the difference observed between B10.HTT (positive) and A.TH (negative). Ia specificities 6 and 7 (20) could account for all the results with this serum, since the lack of reaction with

A.TH means that there is no significant activity in this serum against $H\text{-}2D^d$ antigens (at the antiserum dilution tested [1/150]). Furthermore, the results obtained with serum D-33 suggest that this serum contains another anti-Ia specificity. The serum which was raised against differences in the K and *Ir-1A* regions of B10.A(5R) gave positive reactions with A.TH and B10.HTT. These results could be attributed only to Ia specificity 9, which maps in the *Ir-1A* region, but not to any $H\text{-}2K^b$ antigens. Further investigations along these lines are in progress.

AKR ANTI-θC3H, R ANTI-BAθ, R ANTI-MGG, AND R ANTI-MBLA. Cell surface antigens other than *H-2* can also be detected in the CIA. The following allogeneic and xenogeneic sera were tested: (*a*) AKR anti-θC3H (anti-Thy 1.2) (*b*) R anti-BAθ, (*c*) *R anti-MGG, and* (*d*) R anti-MBLA. These sera were specific for either mouse T lymphocytes (sera *a* and *b*) or for mouse B lymphocytes (sera *c* and *d*) when tested in CDL. When tested in the CIA, strong inhibition was observed with each of these sera (for titers see below). In order to test the specificity of the inhibition of these antisera, we utilized the ability of antibody-coated third-party cells[4] to inhibit cytotoxicity in our system (9). Cells from CBA/H thymus (as a source of T cells) or from nude (nu/nu) mouse spleen cells (as a source of B cells) were incubated with the test sera, washed, and added to the standard cytotoxicity test system. Fig. 2 illustrates the results obtained. While cells pretreated with normal rabbit gamma globulin (NRG) or normal mouse gamma globulin (NMG) resulted in only slight changes in the cytotoxicity, the addition of antibody-coated third-party cells caused inhibition of varying degrees. The specificity of these reactions is represented for each serum by the black and white columns (i.e., inhibition obtained with pretreated T or B cells).

The results generally corresponded with the results from conventional assays, such as CDL or indirect immunofluorescence (see lower part of the figure). Thus, thymus cells inhibited strongly only when pretreated with either of the anti-T-cell sera, whereas nude spleen cells inhibited strongly only when pretreated with either of the anti-B-cell sera. When the third-party cells were complexed with anti *H-2* alloantiserum, the spleen cells showed good inhibition, while the addition of the thymus cells resulted in a surprisingly weak inhibitory effect. This finding may be attributable to differences in the amount of *H-2* antigen expressed on the two cell populations (21).

In summary, many antisera exert specific inhibitory effects when tested in the CIA. The assay detected antibodies directed against (*a*) public and private *H-2D* and *H-2K* specificities (*b*) specificities in the I region which seem to correspond with known Ia specificities, (*c*) at least one non-*H-2* alloantigen (i.e. Thy 1.2; antisera against other non-*H-2* alloantigens have not been tested yet), and (*d*), other cell surface antigens (e.g. immunoglobulin, MBLA).

Sensitivity of the CIA

Unlike most conventional assays, the CIA readily detects antibodies reacting with only a minority of the cells in a heterogenous cell population. This is illus-

[4] The term third-party cells does not imply antigenic differences between these cells and the spleen (effector) cells, but merely that they are exogenous to the latter.

TABLE III
Detection of Ia Specificities in the CIA

Strains tested	*H-2* haplotype	*H-2* regions‡						Ia specificities‡										Inhibition of cytotoxicity§ with antisera‖	
		K	I			S	D											"b anti-5R" (anti-Ia.6, 7)	"D-33" (anti-*H-2K.33*, Ia.9)
			Ir-1A	*Ir-1B*	I-C			1	2	3	4	5	6	7	8	9	10	%	%
B10	b	b	b	b	b	b	b	—	—	3	—	—	—	—	8	9	—	[0]	80 $\mp$ 4***
B10.A(5R)	i5	b	b	b	d	d	d	—	—	3	—	—	6	7	8	9	—	52 $\mp$ 5***	60 $\mp$ 2***
B10.A	a	k	k	k	d	d	d	1	2	3	—	—	6	7	—	—	—	27 $\mp$ 1***	[0]
B10.A(2R)	h2	k	k	k	d	d	b	1	2	3	—	—	6	7	—	—	—	46 $\mp$ 1***	1 $\mp$ 1
B10.A(4R)	h4	k	k	b	b	b	b	1	2	3	—	—	—	—	—	—	—	4 $\mp$ 4	9 $\mp$ 4
B10.HTT	t3	s	s	s	k	k	d	—	—	—	4	—	—	7	—	9	—	46 $\mp$ 3***	59 $\mp$ 2***
A.TH	t2	s	s	s	s	s	d	—	—	—	4	5	—	—	—	9	—	6 $\mp$ 5	67 $\mp$ 3***

‡ See reference 20.

§ See footnote § to Table II for expression of results and significance levels (asterisks); data based on three experiments using the 18-h test (method B); the data were corrected for any inhibition of autologous or parent cells by the test serum; hence these reactions automatically became 0, and are indicated in brackets. The numbers indicate arithmetic means $\mp$ standard deviation.

‖ The sera were tested at a final dilution of 1/150. The specificity of the reactions found with "b anti-5R" can be attributed to anti-Ia.7 (anti-Ia.6 may be present as well). The reactions of D-33 with B10 and B10.A(5R) may be attributable to anti-Ia.9. The anti-Ia.9 activity in a similar serum has been demonstrated recently in a complement dependent assay as well (20).

trated in Table IV. In this experiment spleen cells from BALB/c mice were mixed with spleen cells from CBA/H mice to give effector cell populations with the same total number of cells but with varying proportions of cells from each strain. Upon addition of CBA/H anti-BALB/c serum, inhibition of cytotoxicity could

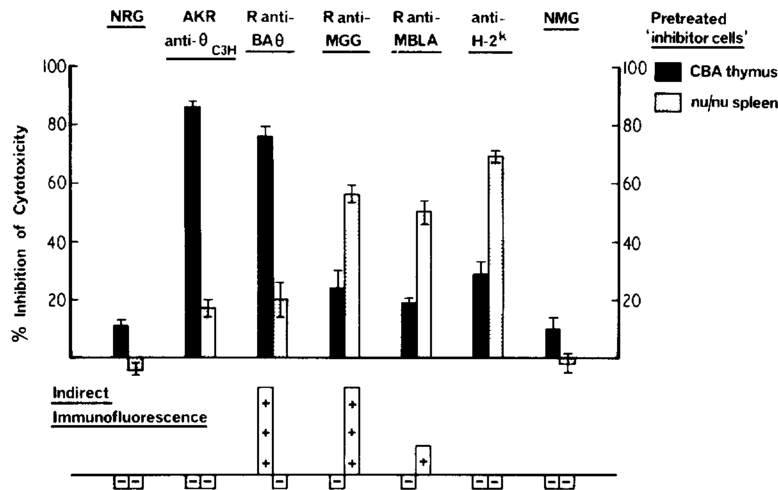

FIG. 2. CIA with preformed antibody-coated "third-party" cells ("inhibitor cells"). CBA/H thymus cells as source of θ-positive cells or nu/nu spleen cells as source of B cells were preincubated for 1 h on ice with the indicated immune or control sera (purified gamma globulin fractions) and washed three times. 10^6 of these cells were added to the standard cytotoxicity test with antibody-coated [^{51}Cr]CRBC as target cells and BALB/c spleen cells as effector cells (method A). In absence of the inhibitor cells cytotoxicity, expressed as percent ^{51}Cr release, was 62%; background ^{51}Cr release without RACA was 2%. The lower part illustrates the results of the indirect immunofluorescence test performed with the same cells using a fluoresceinated goat anti-rabbit gammaglobulin antiserum. +++, intensive stain of all or nearly all cells, +, faint stain of the majority of cells, and —, no specific stain.

TABLE IV

Detection of Subpopulations of Antigen-Bearing Cells by the CIA

Effector cells‡		% Inhibition of cytotoxicity§ in the presence of antiserum‖	
BALB/c	CBA/H	"d anti-k"	"k anti-d"
%	%		
100	0	0 ± 8	94 ± 4***
95	5	50 ± 7**	94 ± 8***
90	10	56 ± 4***	100 ± 4***
50	50	78 ± 5***	94 ± 5***
10	90	83 ± 3***	61 ± 5***
5	95	89 ± 5***	50 ± 8**
0	100	88 ± 5***	13 ± 6

‡ Spleen cells from BALB/c or CBA/H mice were tested either separately or mixed to different proportions for cytotoxicity against antibody-coated ^{51}Cr labeled CRBC (method B).

§ For expression of results and significant levels (asterisks) see footnote § of Table II. The numbers indicate arithmetic means ∓ standard deviation.

‖ The sera were tested at a final dilution of 1/500.

be observed with as few as 5% of the total spleen cells being of BALB/c origin. Similarly, 5% of CBA/H cells in the effector cell mixture could be easily detected with a BALB/c anti-CBA/H serum. Thus, the CIA can easily detect antigens on subpopulations of cells.

An important question relevant to the sensitivity of the CIA was whether the degree of cytotoxicity inhibition by antiserum reflected the total amount of antibody capable of binding to cells in the incubation mixture. To investigate this point, we tested whether there was an additive inhibitory effect in the CIA, when equal aliquots of antiserum, directed either against one or against two different *H-2* specificities expressed on the spleen cells, were added to the incubation mixture. Fig. 3 illustrates an experiment where antisera against speci-

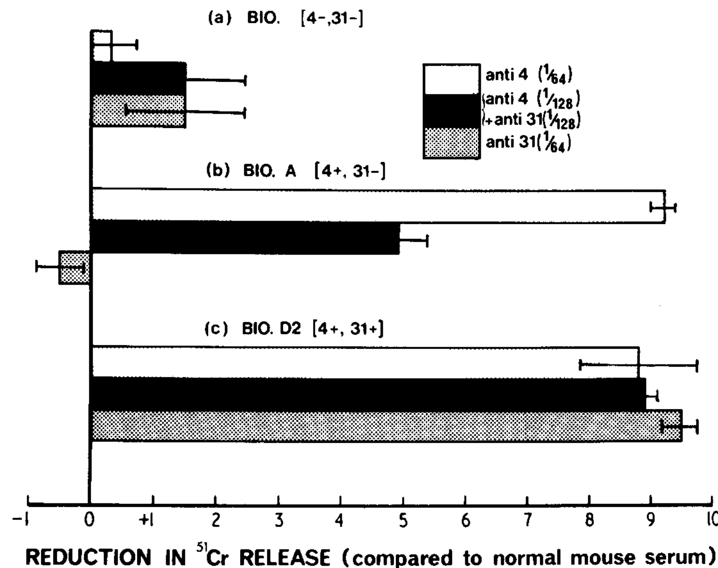

FIG. 3. Additive effect of antisera against different *H-2* specificities in the CIA. The cytotoxicity of spleen cells from three congenic strains of mice (B10, B10.A, and B10.D2) was determined in the presence of the following sera: normal mouse serum (control), "D-4" (*anti-H-2D.4*), "D-31" (anti-*H-2K.31*), or "D-4" plus "D-31". The sera were diluted to give an identical mouse serum concentration (1/64) in all incubation mixtures. For test conditions see method A. The results are expressed as percent ^{51}Cr release with normal serum minus percent ^{51}Cr release with test serum. The horizontal bars indicate the standard deviation of the mean. Background ^{51}Cr release in absence of RACA was 1%.

ficities *H-2D.4* and *H-2K.31* were tested at identical total serum concentrations (1/64) either separately or mixed 1:1, against effector cells from congenic strains negative for both antigens (B10), positive for one antigen (B10.A), or positive for both antigens (B10.D2). The results show (*i*) no significant inhibition of cytotoxicity of B10 cells, (*ii*) specific inhibition of cytotoxicity of B10.A and B10.D2 cells, and (*iii*) additive effects when the amount of antibody was doubled (antibody against one specificity: compare (*b*) second and first column; antibody against two specificities: compare the black columns of *b* and *c*). Thus, the degree of inhibition obtained with a test serum in the CIA seems to reflect the number of antibody molecules binding to cells in the incubation mixture, re-

gardless of the number of antibody specificities. In this respect, the CIA fulfills the prerequisites for a quantitative antibody assay.

The sensitivity of the CIA was investigated for a variety of antisera and compared to the sensitivity of conventional assays (CDL, hemagglutination) (Table V). The inhibition titers represent the highest antiserum dilution which gave significant ($P < 0.01$) inhibition compared to control sera (or to antisera plus autologous cells in the case of anti-*H-2* sera). The data show that the titers for anti-*H-2* sera in the CIA were closely correlated (coefficient of correlation, $r = +0.96$) with the titers in CDL but not with the hemagglutination titers ($r = -0.35$). In every instance, the titers obtained in the CIA were higher (by factors ranging from 2 to 11) than the titers obtained in CDL.

When AKR anti-θC3H, R anti-MBLA, and R anti-MGG were tested in CDL and in the CIA against C3H spleen cells, much higher titers were obtained in the CIA than in the C-dependent test. This difference, which was far greater than that obtained with anti-*H-2* sera, may be due to the fact that the sera were reacting only with subpopulations of the spleen cells. Such reactions may be more difficult to detect in CDL than in the CIA.

In summary, our investigations of the sensitivity of the CIA have shown that the test (*a*) is correlated with but more sensitive than C-dependent antibody assays; (*b*) reflects the number of antibody molecules binding to cell membrane antigens irrespective of whether they react with one or several different antigens; and (*c*) provides a powerful tool for detecting antibody on subpopulations of cells.

Discussion

Cell-dependent antibody-mediated cytotoxicity systems have been described in a number of instances, where antibody-coated target cells were incubated in vitro with nonimmune lymphoid cells. While there is general agreement that the effector cells in these systems are non-T cells which carry Fc receptors, their exact nature is still under discussion (references 22–24 and footnote 5). The antibody-mediating cell-dependent cytotoxicity has usually been found to be of the 7S class (25, 26) and to be thymus dependent (27).

Inhibition of cell-dependent antibody-mediated cytotoxicity has been observed when either immune complexes (9, 28) or aggregated 7S myeloma proteins (8) were added to the system. These reactions seemed to be due to immunoglobulin with altered Fc portions competing with target cell-bound antibody for the Fc receptor of the effector cell. Inhibition has also been observed with antisera directed against cell surface antigens in the effector cell population, usually as an incidental observation, but has never been systematically investigated. In various systems, antisera against immunoglobulin (2, 6, 29), T-cell antigens (30, 31) and HL-A antigens (5) were found to be inhibitory in the absence of C. We have

[5] Pross, H., D. E. Tracey, H. Wigzell, and V. Schirrmacher. 1974. Antibody-dependent cell-mediated cytotoxicity in the mouse. I. Surface characteristics of the effector cell. *Scand. J. Immunol.* In press.

TABLE V

Sensitivity of the CIA Compared to Conventional Assays

Serum*	Strain tested	Control strain	Reciprocal titer‡ in:			Ratio of CIA to CDL
			Hemagglutination§	CDL§	CIA‖	
1 D-4	(B10.D2)	B10.AKM	5,120	320	1,280	4.0
2 D-8	B10.D2	B10	trace	200	1,280	6.4
	B10.BR	B10	trace	56	640	11.4
3 D-23	B10.BR	B10	2,560	240	640	2.6
4 D-25	B10.A(2R)	B10.D2	1,280	170	640	3.8
	B10.BR	B10.A	1,280	370	640	1.7
5 D-31	B10.D2	B10.D2	320	>640	5,120	<8.0
6 D-33	B10	B10.D2	0	2,500	10,240	4.1
7 D-35	B10.D2	B10.A(2R)	1,280	250	640	2.6
8 "k anti-d"	BALB/c	CBA/H	NT	1,280	4,000	3.1
9 "k anti-P815Y"	BALB/c	CBA/H	NT	1,280	4,000	3.1
10 "anti-Thy 1.2"	C3H	AKR	NT	160(40)	6,250	40(156)
11 "R anti-MBLA"	C3H	—	NT	8	6,250	780
12 "R anti-MGG"	C3H	—	NT	320(40)	>6,250	>20(>156)

* See Materials and Methods and Table I.

‡ Highest serum dilution giving a positive ($P < 0.01$) test. The coefficients of correlation between the different assays for sera 1–7 were as follows: between hemagglutination and CDL, $r = -0.27$; between hemagglutination and CIA, $r = -0.35$ (neither of these is significant); between CDL and CIA, $r = +0.96$ (highly significant, $P < 0.001$).

§ Titers for sera 1–7 are taken from the NIH catalogue.[2] CDL was tested in a ^{51}Cr release assay and the titers have been confirmed in our laboratory in the C-dependent microcytotoxicity assay. Sera 8–12 were tested only in the latter assay. Target cells were either lymph node cells (sera 1–9) or spleen cells (sera 10–12). The titers in brackets refer to results obtained with guinea pig C; otherwise rabbit C was used.

‖ Titers were determined in an 18-h assay (method B).

recently reported that anti-*H-2* sera also contain inhibitory activity which is specific and absorbable (10). The inhibition observed with each of these antisera seems to be due to a mechanism similar to the one described above (see paper II).

From these observations we concluded that an assay for antibody could be based on the principle of inhibition of cell-dependent antibody-mediated cytotoxicity. Such an assay system, the CIA, has been described in the present report. The evidence presented indicates that it is capable of detecting antibody against a variety of cell surface antigens in the effector cell population: *H-2* alloantigens mapping in the K, I, and D regions, non-*H-2* alloantigens such as Thy. 1.2, and other antigens detectable by xenogeneic sera, like MBLA and immunoglobulin. As in other systems (32), normal mouse serum had some nonspecific inhibitory activity, which was not completely removed by absorption with autologous cells. For this reason normal mouse serum had to be included as a control in each experiment testing alloantisera. Whenever possible, antisera were tested against autologous or parent cells as an additional negative control.

The antigens detected by the CIA may be identical to the antigens detected in CDL. Both, the specificities of anti-*H-2* sera (Table II) and their titers (Table V) correlated when tested in CDL and CIA. There was, however, no correlation between the titers obtained in CIA and in hemagglutination tests (Table IX). Reasons for this discrepancy are not clear. However, we have observed that (*a*) alloantibody-coated mouse erythrocytes are poorly inhibitory in the CIA even when using alloantisera with strong hemagglutinating activity, and (*b*) mouse erythrocytes have much less *H-2* antigen than mouse lymphocytes when tested in absorbtion experiments. Differences in antibody class and/or in antigen representation could account for the observed discrepancy.

The class of antibody detected in the CIA is under study. From the known specificity of Fc receptors of cytotoxic effector cells for 7S immunoglobulin (see above) it seems likely that the CIA measures only 7S, thymus-dependent antibody. The observed correlation of CDL and CIA in our studies may be due to the fact that we were testing hyperimmune sera only. In early immune sera which contain proportionately more 19S antibody, CDL and CIA may give different results. While CDL and hemagglutination assays measure both 19S and 7S antibody (with different sensitivity [33, 34]), an assay detecting only 7S antibody would have the advantage of being more precise.

The CIA seems particularly valuable in studying membrane antigens expressed only on subpopulations of cells. Even such small proportions as 5% of antigen-positive cells can readily be detected in the CIA (Table IV). This may explain the high titers obtained in the CIA with antisera directed against either B cells or T cells (Table V). Furthermore, the data in Table III suggest that Ia specificities, which are preferentially expressed on B cells (35) can be easily studied with the CIA. The new assay therefore seems to provide a convenient and objective assay for these potentially important specificities.

In addition to its use in identifying membrane antigens on mouse lymphoid cells, the CIA may be applicable to the study of membrane antigens on other cells. We are studying a similar system with human effector cells and human alloantisera and preliminary results suggest that some unexpected specificities may be detectable. The fact that antigens on third-party cells can be tested in the

CIA by precoating them with specific antibody broadens the applicability of the system. For example, cell surface antigens on nonlymphoid cells (e.g., liver and nerve cells) or tumor cells might be investigated in this way. The advantage of such an approach would be (*a*) independence of C, (*b*) no requirement for a purified test cell population, and (*c*) no requirement for radioactive labeling of the test cells.

Summary

Inhibition of cell-dependent antibody-mediated cytotoxicity has been investigated as a new assay for antibody against cell surface antigens. The cytotoxicity system consisted of effector cells (normal mouse spleen cells), target cells (^{51}Cr-labeled chicken erythrocytes), and antitarget cell antibody. Addition of antibody against cell surface antigens in the effector cell population regularly inhibited the cytotoxicity measured in this system. This cytotoxicity inhibition assay (CIA) detected antibody with a variety of specificities: anti-*H-2*, anti-Thy 1.2, anti-immunoglobulin, and antimouse bone marrow-derived lymphocyte antigen. When the inhibition by anti-*H-2* sera was analyzed using effector cells from congenic mice, the activity was found to be directed against specificities mapping in the *H-2K*, *H-2D*, and I regions of the *H-2* complex, correlating well with the specificities characterized by complement-dependent assays.

A comparison between the sensitivity of the CIA and complement-dependent lysis revealed that the CIA was 2–11 times more sensitive for anti-*H-2* antisera and 20–780 times more sensitive for certain antisera against subpopulations of the spleen cells (i.e., T cells or B cells). The CIA proved to be precise, sensitive, and reliable. It may become a very useful antibody assay in various species including man.

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References

1. MacLennan, I. C. M. 1972. Antibody in the induction and inhibition of lymphocyte cytotoxicity. *Transplant. Rev.* **13**:67.
2. Perlmann, P., H. Perlmann, and H. Wigzell. 1972. Lymphocyte mediated cytotoxicity in vitro. Induction and inhibition by humoral antibody and nature of effector cells. *Transplant. Rev.* **13**:91.
3. Zighelboim, J., B. Bonavida, and J. L. Fahey. 1973. Evidence for several cell populations active in antibody dependent cellular cytotoxicity. *J. Immunol.* **111**:1737.
4. Wunderlich, J. R., E. B. Rosenberg, and J. M. Connelly. 1971. Human lymphocyte-dependent cytotoxic antibody and mechanisms of target cell destruction in vitro. *In* Progress in Immunology. D. B. Amos, editor. Academic Press, Inc., New York. 473.
5. Hersey, P., P. Cullen, and I. C. M. MacLennan. 1973. Lymphocyte-dependent cyto-

toxic antibody activity against human transplantation antigens. *Transplantation (Baltimore).* **16:**9.

6. Van Boxel, J. A., J. D. Stobo, W. E. Paul, and I. Green. 1972. Antibody-dependent lymphoid cell-mediated cytotoxicity: no requirement for thymus-derived lymphocytes. *Science (Wash. D. C.).* **175:**194.
7. Dennert, G., and E. S. Lennox. 1973. Phagocytic cells as effectors in a cell-mediated immunity system. *J. Immunol.* **111:**1844.
8. Greenberg, A. H., L. Shen, and I. M. Roitt. 1973. Characterization of the antibody-dependent cytotoxic cell. A non-phagocytic monocyte? *Clin. Exp. Immunol.* **15:**251.
9. Schirrmacher, V., B. Rubin, and H. Pross. 1974. Cytotoxic immune cells with specificity for defined soluble antigens. Analysis of the interaction of antibody with the cytotoxic effector cells in immune or non-immune spleen cells. *J. Immunol.* **112:** 2219.
10. Halloran, P., and H. Festenstein. 1974. Inhibition of cell-dependent cytotoxicity as an assay for mouse alloantibody. *Nature (Lond.).* **250:**52.
11. Rubin, B., and H. Wigzell. 1973. The immune response against hapten-autologous protein conjugates in the mouse, III. Specificity of cooperating non-thymus-processed (B) and thymus-processed (T) lymphocytes. *J. Exp. Med.* **137:**911.
12. Rubin, B. 1973. The immune response against hapten-autologous protein conjugates in the mouse. I. Specificity of antibodies produced during primary response against dinitrophenylated mouse serum albumin. *Eur. J. Immunol.* **3:**26.
13. Baker, A. D., M. S. Rheins, and R. L. St. Pierre. 1973. The effect of rabbit anti-mouse brain associated θ serum on the immunological responsiveness of AKR mice. *Cell. Immunol.* **7:**85.
14. Kerbel, R. S., E. V. Elliott, and V. J. Wallis. 1974. Assessment of mitotic thymus-derived lymphocytes by their sensitivity to the cytotoxic effects of anti-θ serum. *Cell. Immunol.* **11:**146.
15. Raff, M. C., S. Nase, and N. A. Mitchison. 1971. Mouse specific bone marrow derived lymphocyte antigen as a marker for thymus independent lymphocytes. *Nature (Lond.).* **230:**50.
16. Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sussdorf. 1970. *In* Methods of Immunology. W. A. Benjamin, Inc., New York. 2nd edition. 193.
17. Festenstein, H., J. A. Sachs, and R. T. D. Oliver. 1971. Genetic Studies of the mixed lymphocyte reaction in H-2 identical mice. Proceedings of the Symposium on Immunogenetics of the H-2 System, 1970. Liblice, Prague. S. Karger, Basel, Switzerland. 170.
18. Schirrmacher, V., and P. Golstein. 1973. Cytotoxic immune cells with specificity for defined soluble antigens. I. Assay with antigen-coated target cells. *Cell. Immunol.* **9:**198.
19. Demant, P. 1973. H-2 gene complex and its role in alloimmune reactions. *Transplant. Rev.* **15:**164.
20. Shreffler, D. C., and C. S. David. 1974. The H-2 major histocompatibility complex and the I immune response region: genetic variation, function and organisation. *Adv. Immunol.* In press.
21. Aoki, T., U. Hämmerling, E. de Harven, E. A. Boyse, and L. J. Old. 1969. Antigenic structure of cell surfaces. An immunoferritin study of the occurrence and topography of H-2, θ and T1 alloantigens on mouse cells. *J. Exp. Med.* **130:**979.
22. Schirrmacher, V., B. Rubin, P. Golstein, H. Wigzell, and B. Andersson. 1973 Cytotoxic immune cells with specificity for defined soluble antigens. III. Separation from helper cells and from antibody forming cell precursors. *Transplant. Proc.*, **5**(4)**:**1447.

23. Forman, J., and G. Möller. 1973. The effector cell in antibody-induced cell mediated immunity. *Transplant. Rev.* **17**:108.
24. Wislöff, F., and S. S. Fröland. 1973. Antibody-dependent lymphocyte-mediated cytotoxicity in man: no requirement for lymphocytes with membrane-bound immunoglobulin. *Scand. J. Immunol.* **2**:151.
25. MacLennan, I. C. M., C. Loewi, and B. Harding. 1970. The role of immunoglobulins in lymphocyte-mediated cell damage in vitro. I. Comparison of the effects of target cell specific antibody and normal serum factors on cellular damage by immune and non-immune lymphocytes. *Immunology.* **18**:397.
26. Schirrmacher, V., B. Rubin, H. Pross, and H. Wigzell. 1974. Cytotoxic immune cells with specificity for defined soluble antigens. IV. Antibody as mediator of specific cytotoxicity. *J. Exp. Med.* **139**:93.
27. Britton, S., H. Perlmann, and P. Perlmann. 1973. Thymus-dependent and thymus-independent effector functions of mouse lymphoid cells. Comparison of cytotoxicity and primary antibody formation in vitro. *Cell. Immunol.* **8**:420.
28. MacLennan, I. C. M. 1972. Competition for receptors of immunoglobulin on cytotoxic lymphocytes. *Clin. Exp. Immunol.* **10**:275.
29. Henney, C. S., J. Clayburgh, G. A. Cole, and R. A. Prendergast. 1972. B lymphocyte mediated cytolysis: a complement-independent phenomenon. *Immunol. Commun.* **1**:93.
30. Golstein, P., V. Schirrmacher, B. Rubin, and H. Wigzell. 1973. Cytotoxic immune cells with specificity for defined soluble antigens. II. Chasing the killing cells. *Cell. Immunol.* **9**:211.
31. Greenberg, A. H., L. Hudson, L. Shen, and I. M. Roitt. 1973. Antibody-dependent cell-mediated cytotoxicity due to a "null" lymphoid cell. *Nat. New. Biol.* **242**:111.
32. Chia, E., and H. Festenstein. 1973. Specific cytostatic effect of lymph node cells from normal and T cell deficient mice on syngeneic tumor target cells in vitro and its specific abrogation by syngeneic ascites fluid from tumor-bearing mice. *Eur. J. Immunol.* **3**:438.
33. Onoue, K., N. Tanigaki, Y. Yagi, and D. Pressman. 1965. IgM and IgG anti-hapten antibody: hemolytic, hemagglutination and precipitating activity. *Proc. Soc. Exp. Biol. Med.* **120**:340.
34. Borsos, R., and H. J. Rapp. 1965. Hemolysin titration based on fixation of the activated first component of the complement: evidence that one molecule of hemolysin suffices to sensitize an erythrocyte. *J. Immunol.* **95**:559.
35. Hämmerling, G. J., B. D. Deak, G. Mauve, U. Hämmerling, and H. O. McDevitt. 1974. B lymphocyte alloantigens controlled by the I region of the major histocompatibility complex in mice. *Immunogenetics.* **1**:69.