ANTIGEN RECEPTORS ON MAJOR HISTOCOMPATIBILITY COMPLEX-RESTRICTED T LYMPHOCYTES

I. Preparation and Characterization of Syngeneic Antisera against Trinitrophenyl-activated T Cell Blasts and Demonstration of Their Specificity for Idiotypes on Cytotoxic T Lymphocytes*

By PETER H. KRAMMER, RAINER REHBERGER, and KLAUS EICHMANN

From the Institut für Immunologie und Genetik, Deutsches Krebsforschungszentrum, D-6900 Heidelberg, Federal Republic of Germany

Antigen recognition of B cells is a result of the binding of specific membrane Ig to antigens alone, whereas T cell recognition seems to follow different rules. Since the discovery of Zinkernagel and Doherty (1), Shearer et al. (2), and Bevan (3) that mouse cytotoxic T lymphocytes (CTL)[1] activated against virus, the hapten 2,4,6-trinitrophenyl (TNP), and minor histocompatibility antigens recognize the antigens in conjunction with self-histocompatibility determinants, major histocompatibility complex (MHC)- restricted recognition has been regarded as a general phenomenon of the T cell-recognition process. Accordingly, MHC restriction was also seen with helper T cells (T_H), T cells performing delayed-type hypersensitivity (DTH) (T_{DTH}), and T cells proliferating against polypeptide antigens in vitro (4–6).

To explain MHC restriction, two basic models have been proposed: the one-receptor model postulates one single type of T cell receptor (TcR) that binds to neoantigenic determinants (NAD) formed by association of the antigen, e.g., virus, TNP, etc., with MHC antigens, whereas the two-receptor model postulates two different types of TcR, one for the antigen, and the other for syngeneic MHC antigens (7). From the available experimental evidence, a final conclusion on the validity of either one of the two models cannot be made. Therefore, we tried a new approach to this problem by raising antisera against the receptors of H-2-restricted T cells.

* Supported in part by the National Institutes of Health (Bethesda, Md.) contract NO1-CB-74179.

[1] *Abbreviations used in this paper:* a, anti; AKR_{TNBC}, lymph node cells from 2,4,6-trinitrophenylchloride-sensitized mice; AKRa (AKRaAKR-TNP), AKR anti-(AKR anti-trinitrophenylated AKR); aId, anti-idiotypic antiserum(a); ATC, activated T cells; B6, C57BL/6; BId, idiotypes defined by antibodies to B cell receptors; C, complement; CDL, complement-dependent lysis; CML, cell-mediated lympholysis; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; DTH, delayed-type hypersensitivity; FCS, fetal calf serum; FITC, fluorescein isothiocyanate(-conjugated); HBSS, Hanks' balanced salt solution; HS, human serum; Id, idiotype(s); LU_{33}, number of cells per 10^6 cytotoxic effector cells which give 33% lysis in CML with 10^4 target cells per well; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; MLR, mixed-lymphocyte reaction(s); NAD, neoantigenic determinant(s); NMS, normal mouse serum; NP, (4-hydroxy-3-nitrophenyl)acetyl; R, responders; RaMIg, rabbit anti-mouse immunoglobulin antiserum; RBC, red blood cells; S, stimulators; SRBC, sheep red blood cells; TcR, T cell receptor(s); T_{DTH}, T cell(s) performing DTH; T_H, T helper cells; TId, Id defined by antibodies to T cell receptors; TNCB, 2,4,6-trinitrophenylchloride; TNBS, trinitrobenzene sulfonate; TNP, 2,4,6-trinitrophenyl.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/80/05/1166/17 $1.00
Volume 151 May 1980 1166-1182

At the present stage of technology, specific antibodies are the major tool for receptor isolation and characterization. This includes antibodies to variable-region-associated idiotypic determinants. The most common way to prepare anti-idiotype (Id) antisera (aId) is with specific antibodies as immunogens (8), but antigen-specific T cells have also been used to raise aId against TcR (8–12). The latter approach is particularly justified if T cells had different or additional Id (Id defined by antibodies to T cell receptors [TId]) compared to Id on antibodies of the same specificity (Id defined by antibodies to B cell receptors [BId]).

Previously, the method of injecting alloantigen-activated T cell blasts into syngeneic or semisyngeneic recipients has successfully been used to raise aId against Id on alloreactive T cells (11, 12). By following a similar immunization protocol, we describe in this paper a novel system for defining receptors on H-2-restricted AKR anti-(a)AKR-TNP CTL by aId. This new approach may help to elucidate the diversity of the T cell repertoire, the structure of receptors on H-2-restricted T cells, the question of whether the one- or two-receptor model is correct, the identity or nonidentity of TId and BId, and finally, which genes code for antigen receptors on T cells.

Materials and Methods

Animals. AKR/J (H-2^k), C57BL/6 (B6) (H-2^b), and SJL/J (H-2^s) mice were used.

Media. The culture medium was 10 mM of Hepes-buffered RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.), supplemented with L-glutamine (2 mM final concentration), streptomycin (100 μg/ml), 5% human serum (HS), and 2-mercaptoethanol (3×10^{-5} M final concentration). The medium used for the preparation of cell suspensions and for the washing of cells was the same, except that it did not contain 2-mercaptoethanol. The concentration of HS in this medium was 2.5% (wash medium).

Immunofluorescence was performed in Hanks' balanced salt solution (HBSS) with 10% heat-inactivated fetal calf serum (FCS) and 10^{-2} M sodium azide.

Immunization of Mice against 2,4,6-Trinitrophenylchloride (TNCB). Mice were immunized against TNCB by sensitization of the abdominal skin by simultaneous application of 20 μl 30% TNCB in acetone at two different sites in a modification of a procedure that has been described (13, 14). 5 d after immunization, the cells from the draining inguinal and axillary lymph nodes were put into culture.

Immunization of Mice against Fluorescein Isothiocyanate (FITC). Mice were injected intravenously with 2×10^7 irradiated (3,000 rad) FITC-coupled syngeneic spleen cells. Spleen cells from these mice were restimulated in vitro 14 d after immunization by a modification of the procedure of Pohlit et al. (15).

Cell Preparation for Cultures. Lymph node cells from axillary, cervical, inguinal, and mesenteric nodes were prepared and coupled as described previously (16). Mixed lymphocyte reaction (MLR) cultures were set up as described (16); where indicated, responder cells in MLR were enriched for T cells by passing normal lymph node cells over nylon-wool columns (16, 17).

Culture of Cells from TNCB-sensitized Mice. Cells from inguinal and axillary lymph nodes from TNCB-sensitized mice were put into culture at a concentration of 5×10^6/ml in a total vol of 4 ml in 30-ml Falcon bottles (No. 3012; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). The bottles were cultured in an upright position in a 5% CO_2 and 95% air humidified atmosphere.

Restimulation In Vitro of Cells from FITC-immunized Mice. Spleen cells from mice 14 d previously injected with syngeneic FITC-coupled spleen cells (responders [R]) were restimulated in vitro by 3,000-rad-irradiated FITC-coupled syngeneic spleen cells (stimulators [S]) for 5 to 7 days. R were at a cell concentration of 4×10^6/ml, and S at one of 1×10^6/ml (4-ml cultures). The incubation was done in the same way as for cells from TNCB-sensitized mice.

Mitogen Activation. Spleen cells (5×10^6 cells/ml) after lysis of erythrocytes (RBC) were stimulated for 2 d with 5 μg concanavalin A (Con A; No. 15237; C. F. Boehringer and Sons, Mannheim, Federal Republic of Germany) per ml medium.

Preparation of Blasts. Blasts were recovered from cultures by centrifuging 5 ml of cultured cells over a 4-ml Ficoll-Urovison layer (density: 1.077 g/cm^3; Schering-Kahlbaum AG, Berlin, Federal Republic of Germany) in siliconized 12-ml glass tubes at 600 *g* for 15 min at room temperature. Cells (at least 95% viable) were recovered from the interface and washed three times in medium. Usually, 85% of the viable cells obtained from the allogeneic cultures on day 5 were large- and medium-size blasts. Blast cells were defined as cells with a diameter at least twice that of a small lymphocyte, with a smaller nucleus:cytoplasm ratio than small lymphocytes, and with a nonsegmented nucleus (as distinct from nonlymphoid cells).

Trypsin Treatment of T Cell Blasts. The T cell blasts were washed twice in RPMI-1640 with 0.03 M Hepes and 5% heat-inactivated FCS (Gibco Diagnostics, Gibco Invenex Div., Laboratory Park, Chagrin Falls, Ohio), counted, washed once more in RPMI-1640 medium without serum or Hepes, and resuspended in HBSS to a concentration of 2×10^7 cells/ml. An equal volume of phosphate-buffered saline that contained 5 mg/ml tosyl-L-phenylalanyl-chlormethane-treated trypsin (Merck AG, Darmstadt, Federal Republic of Germany) was added to 0.2–1 ml of each cell suspension, and the mixture was then incubated at 37°C for 30 min. Ice-cold RPMI-1640 medium (10 ml) with 15% FCS was added to stop the reaction; the cells were counted, and washed three times. To enable them to recover from trypsin treatment, the T cell blasts were cultured at a concentration of 5×10^5 cells/ml overnight at 37°C, in 5% CO_2 and 95% air humidified atmosphere in fresh culture medium in Falcon flasks (No. 3012).

Determination of [^{3}H] Thymidine Incorporation. 200 μl of cell suspensions from cultures in bottles was transferred in triplicates to round-bottomed microtiter plates (7601305; Linbro Chemical Co., Hamden, Conn.). 2.5 μCi of [^{3}H]thymidine (2 Ci/nmol; Radiochemical Centre, Amersham, England) in 5 μl culture medium was added and [^{3}H]thymidine incorporation over a period of 8 h was determined.

Lysis of RBC. RBC in the pellet from a spleen cell suspension were lysed for 2 min at room temperature by the addition of 1 ml of a solution of nine volumes 0.83% NH_4Cl and one volume 0.17 M Tris (pH 7.5). After lysis, the remaining cells were washed three times and used for mitogen stimulation or immunization.

Coupling of TNP or FITC to Cells. Spleen cells after lysis of RBC were incubated for 10–15 min at 37°C in phosphate-buffered saline (pH 7.2 for trinitrobenzene sulfonate [TNBS] and pH 9.0 for FITC) containing 10 mM TNBS or 100 μg FITC/ml, respectively. After coupling the cells were thoroughly washed three times. When Con A-activated T cells were prepared as target cells for cell-mediated lympholysis (CML), they were first labeled with ^{51}Cr and then with TNP or FITC, respectively.

TNP was coupled to sheep RBC (SRBC) according to Rittenberg and Pratt (18). The number attached to TNP as an index (see below) refers to the amount of TNBS in mg used for coupling (for cells) or the number of TNP groups coupled to a protein.

Standard ^{51}Cr-release Assay. To test the lytic activity of CTL populations we used a standard ^{51}Cr-release assay. Briefly, 100 μl CTL at various cell concentrations were incubated in (Linbro 7601305) round-bottomed microtiter plates with 100 μl ^{51}Cr-labeled Con A-activated target cells. Before incubation, the cells were centrifuged for 5 min at 35 *g*. The incubation time was 4 h at 37°C in a humidified incubator at a 5% CO_2 and 95% air mixture. After the incubation, the supernates from triplicate cultures were harvested after centrifuging the cells for 10 min at 130 *g* and were counted in a γ-counter (Packard, Frankfurt, Federal Republic of Germany). Low controls were taken from cultures with target cells and medium alone. High controls were from cultures frozen and thawed three times. The specific ^{51}Cr-release was calculated according to the formula:

$$\text{specific release (percent)} = \frac{\text{counts per minute experimental release} - \text{counts per minute low control}}{\text{counts per minute high control} - \text{counts per minute low control}} \times 100.$$

The number of cells per 10^6 cytotoxic effector cells which gave 33% lysis in CML with 10^4 target cells per well (LU_{33}) were calculated according to a standard logarithmic curve fit program (Texas Instruments STI-11; Texas Instruments Inc., Digital Systems, Houston, Tex.).

LU_{33} of the NMS control were taken as 0% inhibition to determine the inhibition of CML by CDL with aId.

Normal Mouse Sera (NMS). Normal AKR sera were a pool of sera from 20–30 mice. All sera were heat-inactivated (56°C for 30 min) and absorbed on 2-d Con A-activated spleen cells (100 μl serum and 2×10^7 cells).

aThy-1.1 Antiserum. C3H aThy-1.1 AKR antisera were raised, absorbed, and tested for specificity as previously described (19).

aId. The principle of the production of aId was the injection of enriched TNP-activated AKR T cell blasts intraperitoneally into syngeneic AKR mice. The enriched T cell blasts were washed five times in 10 ml HBSS. At 1- to 4-wk intervals, each mouse received at least three injections. The first injection was in complete, and the subsequent injections were in incomplete, Freund's adjuvant. Individual bleedings from 10–20 mice were pooled. Accordingly, the following sera were raised. First group of mice: (*a*) injection: 7×10^5 cells/mouse; (*b*) injection: 3.2×10^6 cells/mouse (1-wk interval); (*c*) injection: 3×10^6 cells/mouse (2-wk interval). AKR anti-(AKR anti-AKR-TNP) [AKRa (AKRaAKR-TNP)] antisera No. 48 were obtained 3 wk, No. 51 5 wk, and No. 52 6 wk after the third injection. Second group of mice: (*a*) injection: 2.5×10^6 cells/mouse; (*b*) injection: 2×10^6 cells/mouse (2-wk interval); (*c*) injection: 3×10^6 cells/mouse (4-wk interval). Antiserum No. 62 was obtained 5 wk after the third injection.

Absorption of aId. The antisera (at a predetermined dilution in HBSS) were mixed with cells resuspended in a known volume of HBSS. The mixture was incubated for 30 min on ice, the cells centrifuged for 10 min at 16,000 *g*, and the serum was recovered. The dilution was then calculated.

aTNP Keyhole Limpet Hemocyanin (KLH) Antiserum. AKRaTNP-KLH antisera were raised by repeated intraperitoneal injection of 50–100 μg TNP_{178} KLH/mouse into AKR mice. The first injection was in complete, and three additional injections were in incomplete, Freund's adjuvant. Individual bleedings from 15 mice were pooled and tested for hemagglutination on TNP_{50}-SRBC.

Purification of aTNP Antibodies. Briefly, AKRaTNP-KLH antiserum pools with a hemagglutination titer $>10^{-3}$ on TNP_{50}-SRBC were mixed and aTNP antibodies were purified on TNP_{10}-bovine serum albumin-coupled Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) (20).

Absorption of aId on AKRaTNP-KLH Antisera. 100 μl of aId was mixed with 100 μl packed AKRaTNP-KLH-antiserum-coupled Sepharose 4B (0.8 ml of antiserum and 2.7 g of cyanogen-bromide-activated Sepharose 4B). Absorption was performed for 1 h at 4°C. The absorbed aId was recovered by centrifugaton of the mixture at 16,000 *g* for 10 min.

CDL of CTL by aId. 3×10^6 cells from a CTL effector cell population in RPMI 1640 medium that contained 2% FCS were sequentially incubated with the following reagents prediluted in the same medium (*a*) aId (30 min at 4°C), (*b*) rabbit anti-mouse Ig antiserum (RaMIg) (30 min at 4°C), and (*c*) rabbit complement (C) with 10^{-3} M sodium azide (30 min at 37°C). After the first two incubations, the cells were washed twice (10 min at 4°C, 400 *g*), and after the last incubation three times with normal tissue culture medium to remove sodium azide (10 min at room temperature, 400 *g*). Subsequently, the depression of lytic activity versus the NMS control was determined in a standard ^{51}Cr-release assay. aId was used at 1:18 and RaMIg at 1:300 final dilution. Killer:target cell ratios in the Figures and Tables refer to cell counts from the NMS control.

Immunofluorescence. The cells were washed three times in fluorescence medium at 4°C, and were kept throughout in this medium. They were resuspended in 100 μl of medium to which 100 μl of the appropriate antiserum was added (at a predetermined dilution), incubated at 4°C for 30 min, and washed three times between each incubation. After the final antiserum treatment, the cells were washed three times and mounted on glass slides: the preparations were scored under a Zeiss fluorescence microscope (Photomik III; Carl Zeiss, Inc., New York). Staining with the appropriate mouse antiserum was detected by FITC $F(ab')_2$ sheep anti-mouse Ig. The anti-Ig antibodies were raised and conjugated with fluorochromes as described (16), with the modification that affinity-chromatography-purified $F(ab')_2$ fluorescent anti-Ig antibodies were used. All sera as well as fluorescent conjugates were centrifuged at 16,000 *g* for 10 min immediately before use.

Radiolabeling of Antibodies. Radiolabeling with ^{125}I and determination of radioactivity of sheep anti-mouse Ig and AKRaTNP antibodies were performed according to the method of Greenwood et al. (21).

Results

The Experimental System. To raise aId against Id on receptors of H-2-restricted CTL, we chose the following experimental system. AKR mice were immunized against the hapten TNP by sensitization of the abdominal skin with the TNP derivative TNCB. Subsequent (5 d after sensitization) in vitro culture of the cells from the draining axillary and inguinal lymph nodes without antigen restimulation resulted in cell proliferation and the generation of H-2-restricted AKRaAKR-TNP CTL.

Fig. 1 shows that extensive proliferation as detected by [^{3}H]thymidine incorporation and blast-cell count could only be observed in cultures of lymph node cells from immunized, and not from unimmunized, mice. The peak proliferative response in our cultures varied between day 5 and 7 of culture and depended mainly on the batch of HS used as the xenogeneic serum source. Usually, >90% of the cultured cells and >95% of the blast cells were T cells as determined by indirect immunofluorescence with aThy-1.1 antiserum (data not shown).

The time-course of the ensuing cytotoxic response was concordant in magnitude

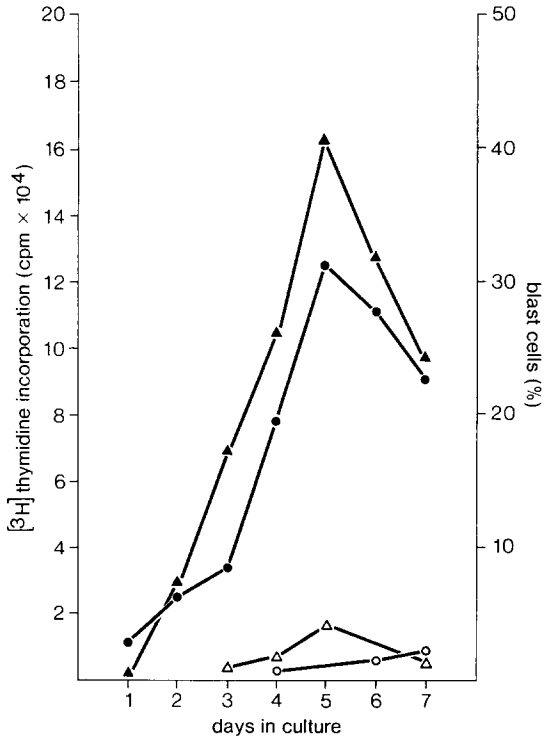

FIG. 1. Proliferation in culture of cells from draining lymph nodes from unimmunized AKR mice and AKR mice sensitized on the abdominal skin by TNCB. Blast cells in cultures of lymph node cells from unimmunized (○), and immunized (●) mice. [^{3}H]thymidine incorporation in cultures of lymph node cells from unimmunized (△), and immunized (▲) mice.

TABLE I

Enrichment of AKRaAKR-TNP CTL from Cultures of Lymph Node Cells from AKR Mice Sensitized by TNCB

Target cells	Percent specific ^{51}Cr release on day 5 of culture*					
	Unseparated		Separated over Ficoll			
			Interphase killer: target ratio		Pellet killer:target ratio	
			10:1	5:1	10:1	5:1
AKR	0	3	6	3	−1	1
AKR-TNP	20	15	42	28	8	5

* CTL: AKRaAKR-TNP.

with blast-cell proliferation (data not shown). Table I shows that AKRaAKR-TNP CTL on day 5 of culture could almost entirely be recovered in the interphase containing viable blast cells after separation of the cultured cells over Ficoll-Hypaque, whereas hardly any CTL could be found in the pellet. CML obtained with the interphase cells was a T cell response, i.e., sensitive to CDL with anti-Thy-1.1 antiserum ([13, 14]; data not shown), with specificity for syngeneic AKR-TNP target cells, i.e., in most experiments, target cells not coupled with TNP or allogeneic TNP-coupled target cells were not lysed (Fig. 2). Occasionally, H-2 restriction was not complete and lysis of B6-TNP target cells with TNCB-sensitized AKR CTL could be observed. In line with experiments reported by Starzinski-Powitz et al. (13) experiments to map the relevant restriction antigens within the H-2 complex indicated that the CML was H-2K^k restricted (P. H. Krammer, R. Rehberger, and K. Eichmann. Manuscript in preparation.).

To raise antisera against Id on receptors of H-2-restricted CTL, we enriched blast cells from AKRaAKR-TNP cultures at the time of peak CTL response and repeatedly injected these cells into syngeneic AKR mice. Antisera were obtained from bleedings starting 3 wk after at least three immunizations and are referred to as AKRa (AKRaAKR-TNP). In an initial screening, we tested the activity of these antisera in indirect immunofluorescence on blast cells enriched for CTL. For our main method of analysis however, we thought it might be particularly important to test AKRa (AKRaAKR-TNP) in functional assays. We therefore used a two-stage CDL test by which we could eliminate CTL. In this assay, cell populations that contained CTL were sequentially incubated with AKRa (AKRaAKR-TNP), RaMIg, and selected rabbit C. RaMIg was used as an enhancing serum in case AKRa (AKRaAKR-TNP) contained non-complement-binding antibodies. This made it necessary to add rabbit C in sodium azide to stabilize the membrane. After CDL, we determined the remaining cytolytic activity of the CTL populations in comparison to CTL populations treated with NMS instead of AKRa (AKRaAKR-TNP).

Reactivity of AKRa (AKRaAKR-TNP) with AKRaAKR-TNP CTL. Tested in indirect immunofluorescence, all AKRa(AKRaAKR-TNP) reacted with AKRaAKR-TNP-CTL-containing cell populations to a similar degree as described in Table IV, lines 3 and 4, i.e., ~70% of all cells were positive with an immunofluorescent intensity that varied from fair to strong, whereas the same antisera showed no immunofluorescence

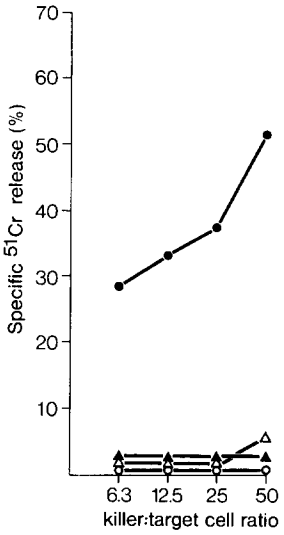

FIG. 2. Specificity of CML by CTL from cultures (day 5) of draining lymph nodes from AKR mice sensitized on the abdominal skin by TNCB on different Con A-activated target cells. AKR (○), AKR-TNP_{10} (●), SJL-TNP_{10} (△), and B6-TNP_{10} (▲) target cells.

TABLE II

Inhibition of CML by Removal of CTL Effector Cells with AKRa (AKRaAKR-TNP) Antisera in CDL

Cell population		CDL of CTL with AKRa (AKRaAKR-TNP) antiserum No.	Inhibition of CML*	Depression of cell numbers after CDL of CTL-containing cells‡
CTL	Target Cells			
			%	%
AKRaAKR-TNP	AKR-TNP	48	57	48
		51	57	33
		52	90	43
		62	91	30
AKRaB6	B6	48	0	0
		52	0	0
		62	0	0
AKRaAKR-FITC	AKR-FITC§	48	0	9
		52	0	10
		62	0	0

* Inhibition of CML after CDL with aId was calculated from LU_{33} of the NMS control taken as 0% inhibition.

‡ Depression of cell numbers after CDL with aId was calculated from the NMS control taken as 0% depression.

§ The data in this group are from separate experiments with reagents that had been distributed in aliquots.

on normal lymph node cells above background with the aIg control. In addition, CDL with all AKRa (AKRaAKR-TNP) consistently depressed between 55 and 95% of TNP-specific AKR CML in comparison to the NMS control. The activity of the antisera resided in the Ig fraction. Table II (lines 1, 2, 3, and 4) and Figs. 3 A, 4 A, and 5 A show the results of representative experiments with the antisera No. 48, 51,

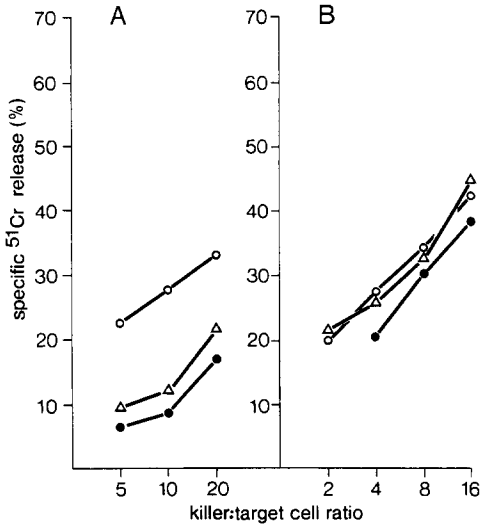

FIG. 3. Specificity of AKRa (AKRaAKR-TNP) No. 48 as tested by CDL on AKRaAKR-TNP (A); and AKRaB6 CTL (B). (○) NMS control, (●) aId No. 48 unabsorbed, and (△) aId No. 48 absorbed on AKRaB6 ATC (50 μl antiserum, 0.8×10^7 ATC).

52, and 62, respectively. It can be seen in Table II that even though the inhibition of CML ranged between 55 and 95% and the number of immunofluorescent-positive cells was ~70% (Table IV), only 30–50% of AKRaAKR-TNP-CTL-containing cells were actually lysed by CDL.

Specificity of AKRa (AKRaAKR-TNP) for AKRaAKR-TNP CTL. Because we had shown that AKRa (AKRaAKR-TNP) reacted with AKRaAKR-TNP CTL, it had to be established whether this reaction had specificity. Therefore, we tested whether AKRa (AKRaAKR-TNP) had activity for other CTL from the same responder strain AKR activated by different antigens, i.e., B6 and AKR-FITC.

It is evident from Fig. 3 that antiserum No. 48 contained a marginal but perhaps significant activity against AKRaB6 CTL which could effectively be absorbed by AKRaB6 MLR-activated T cells (ATC) without a major depression of the relevant antiserum reactivity against AKRaAKR-TNP CTL. Antisera No. 52 and No. 62 were completely nonreactive with AKRaB6 CTL in repeated experiments (Fig. 4B and Table II). The activity of antiserum No. 62 against AKRaAKR-TNP CTL could fully be absorbed by AKRaAKR-TNP-activated cells from day 5 of culture (20 μl antiserum and 10^7 cells) (Fig. 5B), whereas absorption with the same number of AKRaB6 ATC left the activity against AKRaAKR-TNP CTL totally unimpaired (Fig. 5C). Further evidence for nonreactivity of the antisera with alloreactive AKR CTL came from experiments described in Fig. 6. AKR_{TNCB} aB6 CTL obtained from aB6 in vitro activation of cells from draining lymph nodes of mice whose skin had previously been sensitized by TNCB were not lysed in CDL by antisera No. 52 and No. 62 (Fig. 6A and B). These experiments were strongly supported by the following data: CML and actual cell number of AKRaAKR-TNP-CTL-containing cell populations were depressed after CDL with AKRa (AKRaAKR-TNP). This was not the case, however, after the same treatment of AKRaB6-CTL-containing cell populations (Table II).

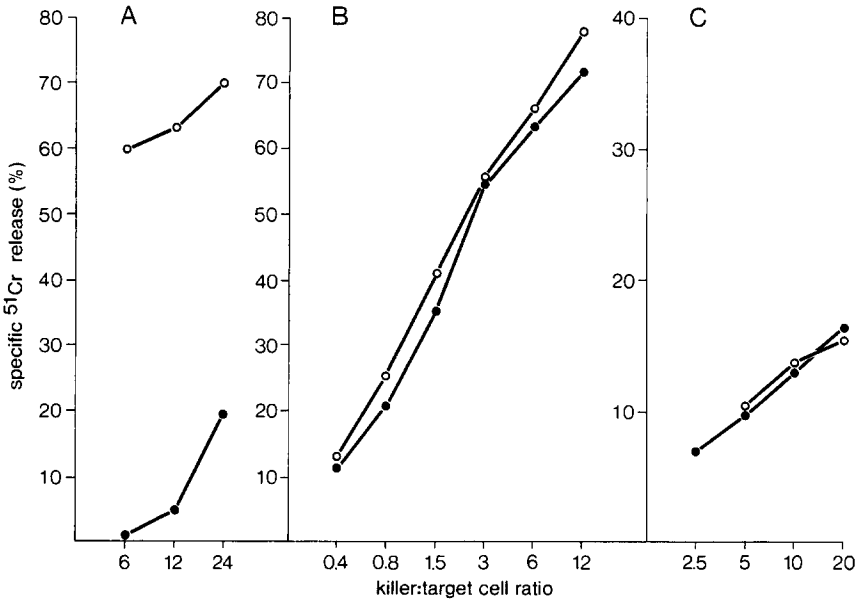

FIG. 4. Specificity of AKRa (AKRaAKR-TNP) No. 52 as tested by CDL on AKRaAKR-TNP(A), AKRaB6(B); and AKRaAKR-FITC CTL (C). (○) NMS control, (●) aId No. 52 unabsorbed.

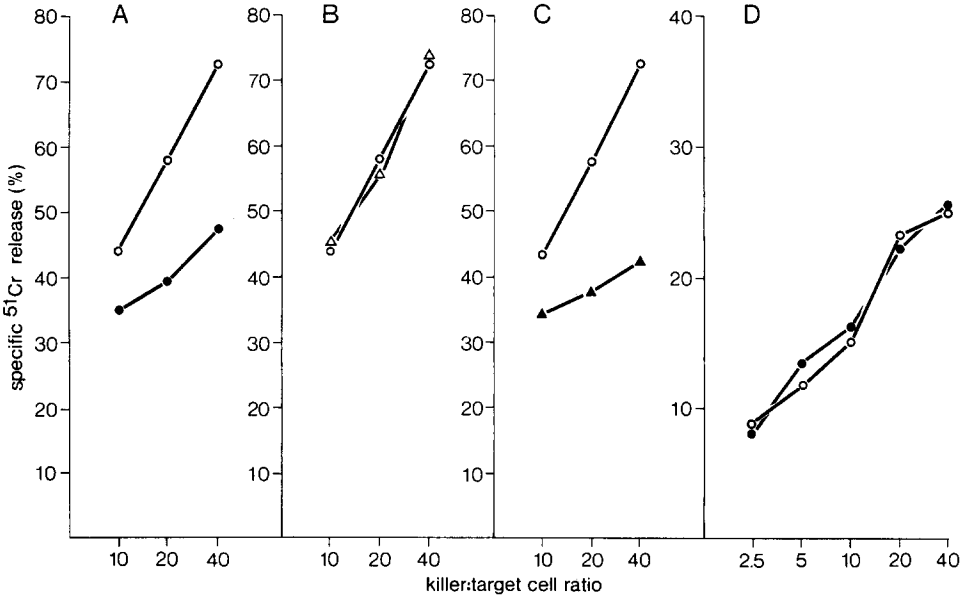

FIG. 5. Specificity of AKRa (AKRaAKR-TNP) No. 62 as tested by CDL on AKRaAKR-TNP (A–C) and AKRaAKR-FITC CTL (D). (○) NMS control, (●) aId No. 62 unabsorbed, (△) aId No. 62 absorbed on AKR cells from day-5 cultures of cells from draining lymph nodes of AKR mice sensitized on the abdominal skin by TNCB (20 μl antiserum, 10^7 cells), and (▲) aId No. 62 absorbed on AKRaB6 ATC (20 μl antiserum, 10^7 cells).

More stringent specificity controls were carried out with FITC-specific AKR CTL. Like AKRaAKR-TNP CTL, AKRaAKR-FITC CTL were anti-Thy-1.1-antiserum sensitive, H-2K^k restricted, and hapten specific. In our hands, in contrast to data from the literature, AKRaAKR-FITC CTL lysed AKR-FITC and not AKR-TNP target

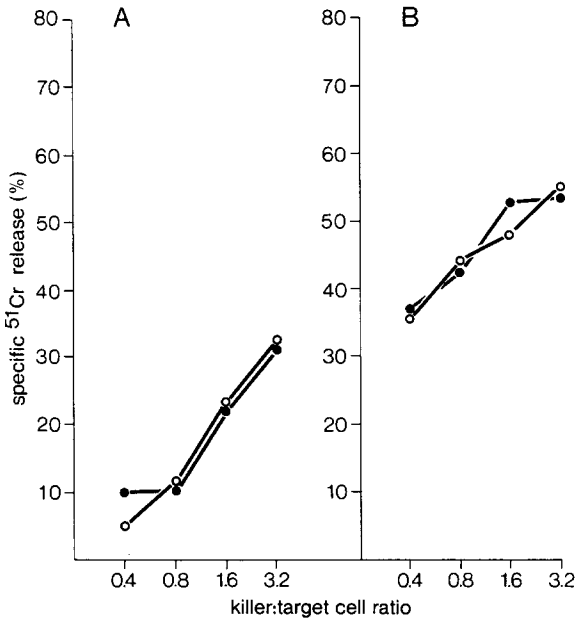

FIG. 6. Lack of reactivity of AKRa (AKRaAKR-TNP) with AKR_{TNCB} aB6 CTL. These CTL were obtained from draining lymph nodes from AKR mice sensitized on the abdominal skin by TNCB and subsequently cultured with B6 stimulator cells. CDL on AKR_{TNCB} aB6 CTL by aId (●), (○) NMS control. (A) aId No. 52. (B) aId No. 62. Target cells in CML were B6.

cells (P. H. Krammer, R. Rehberger, and K. Eichmann. Manuscript in preparation; and [13, 15]). Significantly, as shown in representative experiments, all attempts to demonstrate any appreciable lysis of AKRaAKR-FITC CTL by AKRa (AKRaAKR-TNP) were negative (Table II, antiserum No. 48; Table II and Fig. 4C, antiserum No. 52; Table II and Fig. 5D, antiserum No. 62). These experiments in conjunction with the insignificant depression of cell numbers in AKRaAKR-FITC-containing cell populations after CDL with AKRa (AKRaAKR-TNP) (Table II) showed specificity of the antisera for AKRaAKR-TNP CTL. It should be pointed out that the experiments represented in Figs. 4C, 5D, and 6 were done on a separate occasion. We consider this not critical, however, because for each experiment, different CTL and target-cell populations had to be tested. Furthermore, all reagents were carefully aliquoted and used according to stringent standard conditions. In addition, every experiment was done at least three times with very small variation in the results.

Lack of Reactivity of AKRa (AKRaAKR-TNP) with TNP, TNP-NAD, and Processed CTL-Receptor-bound TNP-NAD. After we had established the specificity of AKRa (AKRaAKR-TNP) for TNP-specific AKR CTL, we concluded that the reactivity of the antisera was directed either against specificity-associated idiotypic determinants or, alternatively, against TNP determinants on AKRaAKR-TNP CTL. TNP determinants could hypothetically be present on CTL membranes in three forms: (*a*) as the hapten TNP itself, (*b*) in association with cell-surface components to create NAD (TNP-NAD), and (*c*) as CTL-receptor-bound TNP-NAD chemically modified by antigen-processing mechanisms.

The following experiments were set up to exclude the possibility that AKRa (AKRaAKR-TNP) had antibody activity against the hapten TNP: the antisera had

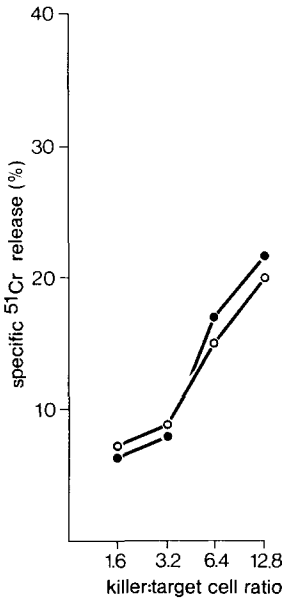

FIG. 7. Lack of reactivity of AKRa (AKRaAKR-TNP) No. 62 with B6aB6-TNP CTL as tested by CDL. (○) NMS control; (●) aId No. 62.

no detectable reactivity with TNP-coupled SRBC in hemagglutination or in radioimmune assays using the antisera and ^{125}I-labeled sheep anti-mouse immunoglobulin IgG as enhancing antibodies (data not shown). Furthermore, lysis of B6aB6-TNP CTL generated in the same way as AKRaAKR-TNP CTL could not be obtained (Fig. 7). In addition, TNP determinants could not be demonstrated by indirect immunofluorescence (Table III) or CDL (data not shown) with highly concentrated, purified AKRaTNP antibodies (hemagglutination titer on TNP_{50}SRBC: 1.64×10^{-4}) on AKRaAKR-TNP CTL that were, however, lysable by AKRa (AKRaAKR-TNP) in CDL.

AKRa (AKRaAKR-TNP) did also not react with the TNP-NAD that might hypothetically have remained on the CTL membrane from the in vivo immunization. Fig. 6 shows that AKR_{TNCB} aB6 CTL effector cells were not lysed. These CTL were obtained by in vitro aB6 stimulation of lymph node cells from mice sensitized in vivo against TNCB under the same conditions as to generate aTNP CTL. Furthermore, indirect immunofluorescence with TNP-coupled AKR lymph node cells was not above the control of Ig-positive cells (data not shown).

Contaminating antibodies against TNP-NAD chemically modified by antigen-processing cells and bound to AKRaAKR-TNP CTL receptors were made unlikely by the results shown in Table IV. Trypsin treatment had efficiently removed CTL receptors and, consequently, any possible remaining processed TNP-NAD. AKRa (AKRaAKR-TNP), however, still lysed AKRaAKR-TNP CTL in CDL after resynthesis of CTL receptors after recovery in overnight culture from trypsin treatment.

These experiments demonstrate that the activity of AKRa (AKRaAKR-TNP) was not directed against TNP. We therefore conclude that the reactivity is directed towards antigenic determinants associated with the specificity of AKRaAKR-TNP CTL. These determinants are most likely of idiotypic nature.

TABLE III

Lack of Detection of TNP Determinants on Cells from Cultures of Lymph Node Cells from AKR Mice Sensitized by TNCB*

Indirect immunofluorescence with	Positive cells‡
	%
AKRa (AKRaAKR-TNP) antiserum No. 62§	72
AKRaTNP antibodies‖	0

* The cells were taken on day 5 of culture.

‡ Percent positive cells from NMS controls (4–5%) were subtracted.

§ The antiserum was used at a dilution of 1:18.

‖ The affinity-chromatography-purified antibodies had a hemagglutination titer on TNP_{50}-SRBC of 1.64×10^{-4}. AKR-TNP_{10} lymph node cells were 100% positive in indirect immunofluorescence with these antibodies.

TABLE IV

Resynthesis of Receptors on Cells from Cultures Containing AKRaAKR-TNP CTL after Recovery from Proteolytic Digestion with Trypsin

Cell population*	Day of culture		Indirect immunofluorescence with AKRa (AKRaAKR-TNP) antiserum No. 62 (percent positive cells)‡	CDL with AKRa (AKRaAKR-TNP) antiserum No. 62 (percent inhibition of CML)§
AKRaAKR-TNP‖	4	Untreated	34	
AKRaAKR-TNP	4	After trypsin treatment¶	0	
AKRaAKR-TNP	5**	Untreated	72	63
AKRaAKR-TNP	5	Recovered from trypsin treatment‡‡	69	75

* Cultures were set up from draining lymph nodes from AKR mice sensitized 5 d previously on the abdominal skin by TNCB.

‡ The percentage of positive cells from the NMS control (4–5%) was subtracted.

§ Inhibition of CML after CDL with aId was calculated from LU_{33} of the NMS control taken as 0% inhibition.

‖ LU_{33} were 416.

¶ Directly after trypsin treatment, no CML could be observed at a killer:target cell ratio of 200:1.

** LU_{33} were 3.

‡‡ LU_{33} were 8 after recovery from trypsin treatment in overnight culture.

Synthesis of Specificity-associated Determinants Detected by AKRa (AKRaAKR-TNP) on AKRaAKR-TNP CTL. To actually show that AKRaAKR-TNP-CTL-specificity-associated determinants detected by AKRa (AKRaAKR-TNP) were synthesized by the CTL themselves we submitted these cells to trypsin treatment to test for resynthesis of such determinants after recovery from enzyme digestion in subsequent overnight culture. Table IV shows that trypsin treatment of a cell population containing AKRaAKR-TNP CTL on day 4 of culture (1 d before peak CML activity) not only depressed the CML activity to background level (Table IV, footnote ¶), but also the number of cells positive in indirect immunofluorescence with AKRa (AKRaAKR-TNP) from 34 to 0%. After overnight incubation (day 5), 69% of cells recovered from trypsin treatment regained immunofluorescent positivity. This number was not greatly different from the number of immunofluorescent-positive cells of a parallel day-5 culture not submitted to enzyme digestion (72%). That untreated CTL as well as

CTL that had recovered from trypsin treatment on day 5 of culture could be lysed almost equally well in CDL by AKRa (AKRaAKR-TNP) (depression of CML: 69 and 75%, respectively) also showed that AKRaAKR-TNP-CTL-specificity-associated determinants were resynthesized by the CTL in <24 h.

It should be pointed out that in this experiment, as in the one described in Table III, the number of immunofluorescent-positive cells by far exceeded the number of cells which could possibly have been accounted for by cells with cytotoxic function.

Discussion

This paper describes a new system to define specificity-associated determinants on TcR of H-2-restricted AKRaAKR-TNP CTL with AKRa (AKRaAKR-TNP) antisera raised by immunizing AKR mice with syngeneic blast cells enriched for AKRaAKR-TNP CTL. Among other possibilities, we chose our experimental system to study Id on H-2-restricted CTL for the following reasons. We tried to limit the number of CTL clones in AKRaAKR-TNP-CTL-containing cell populations used for immunization to more successfully raise aId. Therefore, we used AKRaAKR-TNP CTL, which are restricted mainly to the K end of the MHC, as immunogen. In addition, we assumed that skin sensitization of AKR mice by TNCB with subsequent in vitro development of AKRaAKR-TNP CTL without antigen restimulation provided conditions in which a limited amount of antigen activates a restricted number of CTL precursor clones. Moreover, we tried to reduce the risk of raising antibodies that would contaminate the desired aId activity by using immunization with blast cells purified from dead cells and debris and enriched for AKRaAKR-TNP CTL from cultures to which no TNP had been added. Finally, because aId against alloreactive T cells had successfully been raised by immunization of syngeneic or semisyngeneic recipients with alloantigen-activated T cell blasts (11, 12), we followed a similar protocol and used syngeneic AKR mice as recipients for blast cells from AKRaAKR-TNP-CTL-containing cell populations.

All of our four tested antisera in the TNP system reproducibly demonstrated strong reactivity with a substantial fraction of AKRaAKR-TNP CTL. This suggests that they contained antibodies against a limited number of dominant clonotypes rather than against many different AKRaAKR-TNP CTL clones. In comparison, reproducible activity of aId against alloreactive T cells could, in our hands, only be shown with few antisera and greater experimental difficulties (8). The reason for this could be that alloreactive T cells comprise a heterogeneous mixture of clones with pronounced variation in the dominant Id.

In indirect immunofluorescence and CDL, AKRa (AKRaAKR-TNP) detected between 30 and 70% positive cells in AKRaAKR-TNP-CTL-containing cultures on the day of maximal CML activity. This probably exceeds the number of CTL in these cultures and suggests that the antisera also react with other cells, possibly hapten-specific T_H.

After we established that AKRa (AKRaAKR-TNP) reacted with H-2-restricted AKRaAKR-TNP CTL, we could demonstrate that this activity was specific. In repeated experiments, the antisera did not react with alloreactive AKR CTL, H-2-restricted AKR CTL activated by FITC, and H-2-restricted, TNP-specific CTL of strain B6. This would be in line with the observation that AKRaAKR-FITC CTL in

our hands are not cytotoxic for AKR-TNP target cells and apparently have TcR with combining sites different from AKRa AKR-TNP CTL.

Because AKRa (AKRaAKR-TNP) detected specificity-associated determinants on AKRaAKR-TNP CTL, it remained to be shown whether this activity was directed against idiotypic determinants on AKRaAKR-TNP CTL TcR or against the antigen TNP that might hypothetically have persisted on AKRaAKR-TNP CTL membranes. We could exclude the possibility that AKRa (AKRaAKR-TNP) reacted with the antigen TNP alone, TNP in association with lymphocyte-surface components creating TNP-NAD, or putative CTL-receptor-bound TNP-NAD chemically modified by antigen-processing mechanisms. We concluded therefore, that the relevant antibodies in AKRa (AKRaAKR-TNP) were aId-directed against the idiotypic determinants on receptors of H-2-restricted AKRaAKR-TNP CTL effector cells.

There are two major arguments to be raised against the firm conclusion that our antisera have specificity for Id. Both arguments concern the fact that the control CTL, which did not react with our antisera, were produced by immunization protocols that differ from that used to raise AKRaAKR-TNP CTL. This leads to the possibility that in addition to different idiotypes, either different differentiation antigens (viruses, blasts, etc.) are expressed on the different CTL populations, or that pronounced differences exist in clonal idiotypic heterogeneity. In the latter case, even the use of the same hapten as stimulator in two different immunization procedures could lead to absence of idiotypic cross-reactivity between the two CTL populations. With respect to differentiation antigens, we came to the conclusion that no biological experiment exists to formally rule out this possibility. We should point out, however, that in many similar immunization protocols, antisera were obtained that were formally shown to react with T cell receptors (11, 12). In the absence of the strong experimental evidence of cross-reactivity with antibodies of the same specificity (see below), we shall have to use biochemical procedures to eliminate the possibility of a differentiation antigen. Such experiments are in progress. Possible differences in clonal heterogeneity between FITC- and TNP-specific CTL raised by two different protocols could lead to the situation that a small number of FITC-specific CTL clones would express full reactivity with our antisera but would escape detection in the very heterogeneous mixture of FITC-specific clones. In this case, we would have to conclude that although our antisera do not distinguish between some TNP-specific binding sites and some FITC-specific binding sites, they do distinguish between various different FITC-specific binding sites. Also in this case the specificity of the antisera had to be defined as idiotypic.

It has previously been shown that aId raised against alloreactive T cell blasts could partially be absorbed by alloantibodies of the same specificity, indicating that at least a fraction of TId was overlapping with BId (11). We set up similar experiments to test whether Id defined on H-2-restricted AKRaAKR-TNP CTL TcR by AKRa (AKRaAKR-TNP) were also present on AKRaTNP antibodies. We have extensively absorbed AKRa (AKRaAKR-TNP) on a pool of high-titered AKRaTNP-KLH antisera to subsequently determine the decrease of aId activity on AKRaAKR-TNP CTL in CDL and indirect immunofluorescence. We have tried to competitively inhibit (*a*) CDL by AKRa (AKRaAKR-TNP) of AKRaAKR-TNP CTL by addition of variable amounts of AKRaTNP antibodies and (*b*) hemagglutination by AKRaTNP-KLH antiserum of TNP_{50}-SRBC by addition of variable amounts of aId.

Finally, we tested the binding of ^{125}I-labeled AKRaTNP antibodies to aId-coated microtiter plates (22). The results of these experiments were negative (data not shown). This could be explained in a number of different ways. A minor fraction of overlapping TId and BId might have escaped our detection or overlapping TId and BId might be expressed at different time points of the immune response. On the other hand, to be able to detect overlapping TId and BId, it might be essential to raise the relevant antibodies by skin sensitization of AKR mice with TNCB instead of immunization with TNP-KLH. This has also been extensively tried but the amount of aTNP antibodies obtained by that procedure was negligible. We think that the lack of reactivity with aTNP antibodies is presently not a serious argument against the idiotypic specificity of our antisera and the question of identical or nonidentical Id repertoire of T and B cells needs to be resolved by further experiments. Id on TcR of H-2-restricted CTL (TId) and Id on antibodies (BId) may be similar or nonoverlapping, depending on the presentation of the immunizing hapten. One could infer this from a comparison of the data by Ando and Kisielow (23) and by Weinberger et al. (24): even though primary anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) antibodies from H-2^b mice are heteroclitic, i.e., they react with higher affinity with the hapten (4-hydroxy-5-iodo-3-nitrophenyl)acetyl (NIP), NP-specific CTL from the same strain do not have this Ig-1-allotype-linked, fine-specificity marker that is characteristic for the Ig variable region of the heavy chain. In contrast, NP-specific, DTH-reactive T cells of B6 mice were shown to possess similar heteroclicity as anti-NP antibodies. The responsible difference between the two systems may lie in the presentation of the antigen, which was NP on lymphocytes for CTL induction, but NP on soluble proteins for DTH and antibody induction.

The data in this paper have to be discussed with respect to expression on AKR CTL of one type of receptor for TNP-self-antigens (one-receptor model), two types of receptors for the hapten TNP and AKR MHC antigens (two-receptor model), or one complex receptor type with two combining subsites (a combination of the one- and two-receptor model). Different sets of single or complex Id could be expressed in each case. That AKRa (AKRaAKR-TNP) did not detect TcR on H-2-restricted AKRaAKR-FITC CTL would either support a one-receptor model or could be interpreted in the sense that putative anti-self receptors are not immunogenic because of their representation on a large proportion of all T lymphocytes and are therefore not recognized by the antisera. A tentative answer to this question could be provided by testing our antisera on AKRaTNP CTL from chimeric mice. These serological experiments may give an indication, but a final decision on the validity of either one of the above models will eventually come from biochemical and genetic experiments.

To this end, the fact that Id detected by AKRa (AKRaAKR-TNP) show strain specificity (e.g., AKR CTL are positive and B6 CTL are negative) will enable us to study the genetic control of Id expression on H-2-restricted CTL. In conclusion, the TNP CTL idiotype system provides us with a new tool to study T cell recognition, the biochemistry of TcR, and the regulation of the generation of H-2-restricted CTL on the Id level.

Summary

This paper describes the specificity of AKR anti- (a) [AKR anti-trinitrophenylated AKR (AKR-TNP)] [AKRa (AKRaAKR-TNP)] antisera raised in syngeneic AKR

mice against AKRaAKR-TNP cell populations enriched for H-2-restricted aTNP cytotoxic lymphocytes (CTL) by blast-cell isolation. The activity of the antisera resided in the Ig fraction. All antisera were shown to reproducibly react with AKRaAKR-TNP-CTL-containing cell populations in indirect immunofluorescence and all removed the major fraction of CTL in complement-dependent lysis causing a considerable depression of cell-mediated lympholysis. The antisera were nonreactive with alloreactive AKRaC57BL/6 CTL and other H-2-restricted AKR CTL against fluorescein-isothiocyanate-conjugated AKR- target cells. It could be excluded that the antisera contained contaminating antibodies against TNP, TNP-neoantigenic determinants (NAD), or processed CTL-receptor-bound TNP-NAD, thus demonstrating specificity for determinants on T cell receptors of AKRaAKR-TNP CTL. These receptors were produced by the CTL themselves.

These observations are interpreted to suggest that AKRa (AKRaAKR-TNP) antisera contain anti-idiotypic antibodies directed against specificity-associated determinants (idiotypes) on T cell receptors of H-2-restricted AKRaAKR-TNP CTL. The antisera provide a new tool to study the genetic control of idiotype expression on H-2-restricted CTL, the biochemistry of T cell receptors, and the regulation of the generation of H-2-restricted CTL on the idiotype level.

We thank Mrs. Doris Mohr for helpful technical assistance, and Doctors Wulf Droege, Ulrich Koszinowski, Volker Schirrmacher, and Markus Simon for critical discussions.

Received for publication 14 January 1980.

References

1. Zinkernagel, R. M., and P. C. Doherty. 1975. *H-2* compatibility requirement for T-cell-mediated lysis of targets infected with lymphocytic choriomeningitis virus. Different cytotoxic T-cell specificities are associated with structures coded in *H-2K* or *H-2D*. *J. Exp. Med.* **141**:1427.
2. Shearer, G. M., T. G. Rehn, and C. A. Garbarino. 1975. Cell-mediated lympholysis of trinitrophenyl-modified autologous lymphocytes. Effector cell specificity to modified cell surface components controlled by the *H-2K* and *H-2D* serological regions of the murine major histocompatibility complex. *J. Exp. Med.* **141**:1348.
3. Bevan, M. J. 1975. The major histocompatibility complex determines susceptibility to cytotoxic T cells directed against minor histocompatibility antigens. *J. Exp. Med.* **142**:1349.
4. Sprent, J. 1978. Role of the H-2 complex in induction of T helper cells in vivo. I. Antigen-specific selection of donor T cells to sheep erythrocytes in irradiated mice dependent upon sharing of H-2 determinants between donor and host. *J. Exp. Med.* **148**:478.
5. Miller, J. F. A. P., M. A. Vadas, A. Whitelaw, and J. Gamble. 1976. Role of major histocompatibility complex gene products in delayed-type hypersensitivity. *Proc. Natl. Acad. Sci. U. S. A.* **73**:2486.
6. Yano, A., R. H. Schwartz, and W. E. Paul. 1977. Antigen presentation in the murine T-lymphocyte proliferative response. I. Requirement for genetic identity at the major histocompatibility complex. *J. Exp. Med.* **146**:828.
7. Zinkernagel, R. M., G. N. Callahan, A. Althage, J. Cooper, P. A. Klein, and J. Klein. 1978. On the thymus in the differentiation of H-2 self-recognition by T cells: evidence for dual recognition? *J. Exp. Med.* **147**:882.
8. Eichmann, K. 1978. Expression and function of idiotypes on lymphocytes. *Adv. Immunol.* **26**: 195.
9. Binz, H., and H. Wigzell. 1976. Specific transplantation tolerance induced by autoimmu-

nization against the individual's own, naturally occurring idiotypic, antigen-binding receptors. *J. Exp. Med.* **144:**1438.

10. Ramseier, H., and J. Lindenmann. 1972. Similarity of cellular recognition structures for histocompatibility antigens and of combining sites of corresponding alloantibodies. *Eur. J. Immunol.* **2:**109.
11. Krammer, P. H. 1978. Alloantigen receptors on activated T cells in mice I. Binding of alloantigens and anti-idiotypic antibodies to the same receptors. *J. Exp. Med.* **147:**25.
12. Andersson, L. C., H. Binz, and H. Wigzell. 1976. Specific unresponsiveness to transplantation antigens induced by autoimmunization with syngeneic, antigen-specific T lymphoblasts. *Nature (Lond.).* **264:**778.
13. Starzinski-Powitz, A., K. Pfitzenmaier, M. Röllinghoff, and H. Wagner. 1976. In vivo sensitization of T cells to hapten-conjugated syngeneic structures of the major histocompatibility complex I. Effect of in vitro culture upon generation of cytotoxic T lymphocytes. *Eur. J. Immunol.* **6:**799.
14. Droege, W., W. Suessmuth, R. Franze, P. Mueller, N. Franze, and J. Balcarova. 1979. Suppressor cells regulating the in vivo induction of cytotoxic T lymphocytes and of memory cells for a secondary in vitro CMC response. *In* Cell Biology and Immunology of Leukocyte Function. M. R. Quastel, editor. Academic Press, Inc., New York. 467
15. Pohlit, H., W. Haas, and H. von Boehmer. 1979. Cytotoxic T cell responses to haptenated cells. I. Primary, secondary and long-term cultures. *Eur. J. Immunol.* **9:**681.
16. Krammer, P. H., R. Citronbaum, S. E. Read, L. Forni, and R. Lang. 1976. Murine thymic lymphomas as model tumors for T cell studies. T-cell markers, immunoglobulin and Fc-receptors on AKR thymomas. *Cell. Immunol.* **27:**97.
17. Julius, M., H. E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived lymphocytes. *Eur. J. Immunol.* **3:**645.
18. Rittenberg, M. B., and K. L. Pratt. 1969. Anti-TNP plaque assay-primary response of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* **132:**575.
19. Wettstein, P. J., P. Krammer, R. C. Nowinski, C. S. David, J. A. Frelinger, and D. C. Shreffler. 1976. A cautionary note regarding Ia and H-2 typing of murine lymphoid tumors. *Immunogenetics.* **3:**507.
20. March, S. C., J. Parikh, and P. Cuatrecasas. 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. *Anal. Biochem.* **60:**149.
21. Greenwood, F. C., W. H. Hunter, and J. S. Glover. 1963. The preparation of ^{131}I-labeled growth hormone of high specific activity. *Biochem. J.* **89:**114.
22. Klinman, N. R., A. R. Pickard, N. H. Sigal, P. J. Gearhart, E. S. Metcalf, and S. K. Pierce. 1976. Assessing B cell diversification by antigen receptor and precursor cell analysis. *Ann. Immunol. (Paris).* **127C:**489.
23. Ando, J., and P. Kisielow. 1979. Fine specificity of cytotoxic T lymphocytes: C57BL effector cells induced by autologous cells modified with hapten (4-hydroxy-3-nitro-phenyl)acetyl (NIP) are not heteroclitic. *Eur. J. Immunol.* **9:**211.
24. Weinberger, J. Z., M. I. Greene, B. Benacerraf, and M. E. Dorf. 1979. Hapten-specific T-cell responses to 4-hydroxy-3-nitrophenyl acetyl. I. Genetic control of delayed-type hypersensitivity by V_H and I-A region genes. *J. Exp. Med.* **149:**1336.