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K-cell-mediated cytotoxicity induced with rat monoclonal antibodies
I. ANTIBODIES OF VARIOUS ISOTYPES DIFFER IN THEIR ABILITY TO INDUCE CYTOTOXICITY MEDIATED BY RAT AND HUMAN EFFECTORS

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SUMMARY

Among the rat antibodies tested in this study, monoclonals of IgG2a isotype were the best, IgG1 and IgG2b being also effective, for induction of K-cell-mediated cytotoxicity of relevant nucleated target cells by rat effectors (Nude spleen cells). The single rat monoclonal IgE tested was also active. Rat antibodies of IgM and IgA classes of immunoglobulins were not active in that system. IgG2c and IgD monoclonals were not tested. Using the same pannel of reagents, cytotoxicity mediated by human K cells (peripheral blood mononuclear cells) was only induced by the IgG2b class of rat monoclonals.

INTRODUCTION

K cells are mononuclear cells known for their cytotoxic potential that can be assessed *in vitro*. They require a low amount of antibody to lyse sensitized 'target' cells.

The description of K cells reviewed by MacLennan (1972) has been largely confirmed by many later investigators, but some essential points remain unknown: What is the role of K cells? How and when are they triggered *in vivo*? Could they be used in a controlled fashion, *in vitro* (to destroy a cell subpopulation for instance), or even *in vivo*?

The *in vitro* measure of K-cell cytotoxicity in various pathological situations has been performed extensively without giving definite clues about the possible protective role of K cells, nor about the role they may have in induction of pathologies. On the other hand, some reports point towards their possible regulatory role. They may lyse hybridoma cells or plasmacytoma cells when sensitized with respective anti-idiotypes (Caraux & Weigle, 1983; D. Chassoux and M. Stanislawski, unpublished observations). They may be involved in bone marrow graft rejection (Warner & Dennert, 1985). The mode of death of target cells after a K-cell attack is that of apoptosis, the endogenous mode of cell death that seems to be part of homeostatic regulation (Duvall & Wyllie, 1986). Clearly, further studies are needed and we therefore undertook a systematic analysis of some parameters of K-cell cytotoxicity. In the rat model, chosen for experimental situations as explained before (Chassoux *et al.*, 1983b), K cells do not need either the thymus or the spleen to develop (Chassoux *et al.*, 1983a; Chassoux, Kolb & MacLennan, 1985) and they are likely to represent an independent cell lineage from that of T and B cells (Chassoux *et al.*, 1983b).

Here, we studied comparatively antibodies of various isotypes in order to determine if some isotypes would be used preferentially by K cells to lyse appropriate target cells. We chose rat Ab to be in homologous situations with rat effector cells. We also examined the ability of human effectors to work with rat mAbs in a K-cell assay, bearing in mind the possible therapeutic applications of such a system.

Monoclonals represent a unique possibility for this study. It is now possible to obtain large amounts of Ab of a given isotype without contamination by Ab of another isotype. The purification system chosen here avoided the differences in affinity of Ab for the antigen. Moreover, all antibodies used in this study were directed against the same antigen and we were able to test a number of antibodies of a given isotype (i.e. monoclonals obtained in different instances).

MATERIALS AND METHODS

Monoclonal antibodies

MAbs used in this study are listed in Table 1. Hybridoma cells were constructed by fusion of IR983F with hyperimmune rat spleen cells. The LOU rat immunocytoma IR983F is non-secreting and azaguanine resistant (Bazin, 1982). Fusion of IR983F with spleen cells was performed according to Declerq, Cormont & Bazin (1986). Positives cells were recorded by ELISA on BGG-DNP-coated plastic wells. Anti-DNP secreting hybridoma cells were cloned, expanded and grafted into congenic LOU/C IgK1b (OKA) rats (Bazin, Beckers & Querin-

Abbreviations: Ab, antibody; BGG, bovine gamma globulin; DNP, 2,4 dinitrophenyl; FCS, fetal calf serum; mAb, monoclonal antibody; SC, specific cytotoxicity; TNBS, 2,4,6 trinitrobenzene sulphonic acid; TNP, 2,4,6 trinitrophenyl.

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Table 1. Monoclonal antibodies listed according to their isotype*

IgM	IgG1	IgG2a	IgG2b	IgA	IgE
LO-DNP-34	LO-DNP-1	LO-DNP-3	LO-DNP-11	LO-DNP-44	LO-DNP-30
LO-DNP-39	LO-DNP-2	LO-DNP-4	LO-DNP-48	LO-DNP-45	
	LO-DNP-9	LO-DNP-7	LO-DNP-55	LO-DNP-47	
	LO-DNP-15	LO-DNP-16	LO-DNP-56	LO-DNP-49	
	LO-DNP-22	LO-DNP-20	LO-DNP-57	LO-DNP-51	
			LO-DNP-62	LO-DNP-68	
			LO-DNP-66		

* Antibodies of IgD and IgG2c classes were not available for testing.

jean, 1974) for ascitis production (Bazin, Cormont & Declercq, 1984a).

Monoclonal antibodies were used as ascitic fluids or purified antibodies. The purification was done following the method described by Bazin *et al.* (1984a). Briefly, mAbs directed against rat kappa 1a allotype (MARK 3), were purified from BALB/c mouse ascitis by immunoaffinity using an IgG1 (Kappa 1a) myeloma rat protein (Bazin *et al.*, 1984b) column. An immunoabsorbent was prepared by coupling purified MARK 3 to Sepharose 4B (Pharmacia, Uppsala, Sweden). Ascitic fluids were passed on such columns and antibodies eluted with glycine-HCl 0·1 M. Eluates were dialysed twice against PBS at 4°. Antibodies' concentrations were estimated from OD measures at 280 nm.

Target cells

Chang cells were purchased from Gibco (Grand Island, NY) and maintained in the laboratory. This is an adherent cell line which may be grown in S-MEM (Gibco no. 0421665H) supplemented with antibiotics, glutamin, non-essential amino acids, bicarbonate and 5% heat-inactivated fetal calf serum (FCS). A proportion of cells then detach from the monolayer in the supernatant and may be collected by simple shaking of the culture flask. Medium was always renewed less than 24 hr before an assay.

Hapten modification of Chang cells was performed as described by Shearer (1974). ^{51}Cr-labelled cells were washed in PBS and incubated for 10 min at 37° in TNBS (Serva, Heidelberg) (10 mM)-PBS, pH 7·3. Cells (Chang-TNP) were then washed twice in MEM with 10% FCS added and resuspended in RPMI-1640 (Gibco) supplemented with antibiotics, glutamin, pyruvate and 10% FCS (RPMI-10).

Effector cells

Rat effectors. Outbread adult rnu/rnu rats, 3–4 months old, were submitted to ether anaesthetics. Spleens were removed and spleen cells suspension were prepared by forcing splenic tissue through a stainless steel mesh. Cells were suspended in MEM, washed twice and resuspended in RPMI-10 at 10^7 mononuclear cells per ml. Cells from several animals were used individually and never pooled. In some instances, effector cells were enriched before use. This was done by separation of mononuclear cells from polymorphs and red cells on Ficoll gradient. Cells were then washed and incubated on a plastic surface for 2 hr at 37° in the presence of FCS, in order to remove adherent cells. Non-adherent cells were recovered and washed once before use.

Human effectors. Peripheral blood from volunteers was obtained from the Transfusion Centre, Hospital Paul Brousse, Villejuif, France. Heparinized blood was diluted in MEM and layered over a Ficoll gradient (Lymphoprep, Nyegaard, Norway).

Cells from the interface was collected, washed three times in MEM +2% FCS, resuspended at $1–2 \times 10^6$ cells/ml in RPMI-10 and cultured overnight in 250-ml culture flasks. Adherent cells stuck to the plastic surface and non-adherent cells were collected, washed twice and resuspended in RPMI-10.

Cytotoxicity assays

Antibodies to be tested were serially diluted in 50 μl of RPMI-10 in round-bottomed wells of Microtitre plates (Greiner, Postfach, FRG). Targets (10^4) were added under a volume of 100 μl and effectors (50 μl) were distributed last at ratios indicated in the results section. Usually effectors from Nude rats were used at 50 spleen mononuclear cells for one target. Human effectors were used at an effector:target cell ratio of 20–30:1. Five-hour incubations were performed at 37° in an incubator adjusted at 5% CO_2 in air.

Half of the supernatants were then collected and transferred to plastic tubes for counting in an LKB gamma counter (60 seconds per tube). Samples of total radioactivity (RT) corresponding to 10^4 ^{51}Cr-labelled cells were always counted. Specific cytotoxicity values (SC) were obtained from (percentage release BL/maximum release − BL) × 100, where percentage release is (observed c.p.m. × 2/RT) × 100, BL is the percentage ^{51}Cr release from targets alone, maximum release being the percentage of label released by targets incubated with 10% perchloric acid.

Incubation of targets with effectors alone (no antibody) was always included in an assay (NK), as well as a positive control, usually a rat polyclonal anti-DNP previously tested.

Chang-TNP, as Chang cells, remain relatively resistant to rat NK cells (10–15 SC), whereas they display an increased sensitivity to human NK cells (up to 30 SC). Chang cells are normally a poor target for human NK (10–15 SC). Results are given as mean of duplicate determinations. Titre refers to a specific cytotoxicity value of 10% above lysis in absence of antibody (i.e. NK activity).

Cellular radioimmunoassays

Chang-TNP were distributed in flat-bottomed flexible assay plates (Falcon, Becton-Dickinson, Switzerland) at 2×10^5/ml in RPMI-10 and left to adhere on the plastic surface for 4 hr at 37°. After two washings in MEM at room temperature done by

Table 2. Maximum K-cell-specific cytotoxicity levels recorded using ascitis mAbs and rat effectors

	MAb no. (as in Table 1)	Experiments 1	2	3	4	5	6*
IgG2a	7		48	76		46	42
	3	56		59			
	16				64		
	20			60			30
	4	47		60			
IgG2b	55			59		33	
	56					29	
	57			60		24	
	62					19	
	66					18	
	48					18	
	11		33			21	14
IgG1	1	43				29	
	2	46				20	
	9		37				27
	22		40				27
	15			44			19
IgE	30			51	47	30	19
NK	—	15	15	13	15	9	11

* LOU/C rat effectors were used in that experiment.

flicking the plates, cells were fixed with 0·25% glutaraldehyde at 0° for 5 min and washed three times with PBS. The wells were then covered with 0·5% BSA/PBS and left overnight at 4°. Assays were performed the next day. Plates were brought to room temperature, flicked and serial dilutions of samples to be tested distributed. Incubation was run for 2 hr and after two washings in PBS, ^{125}I MARK 1 added (mouse monoclonal anti-rat kappa) (Bazin *et al.*, 1984b). A 2-hour incubation at room temperature was done before extensive washings. Retained label was measured in a gamma counter by placing the cut wells in tubes. A standard curve was established with a sample of known concentration, and unknown calculated from the best fit to the curve.

RESULTS

Rat K-cell cytotoxicity of Chang-TNP target cells sensitized with anti-DNP rat mAbs

Anti-DNP rat monoclonal antibodies of IgG isotype. MAbs were used as ascitic fluids from LOU rats. Seventeen different mAbs of IgG1, IgG2a and IgG2b isotypes were tested. Most of these were assayed twice or more over seven separate experiments. In all instances K-cell-mediated lysis was obtained. In Table 2, maximum levels of specific cytotoxicity recorded for each Ab are indicated. One can see that, within each experiment, SC values higher than those achieved with IgG2a mAbs were never found in groups of antibodies of the other isotypes, so that mAbs of IgG2a isotype are apparently inducing K-cell-mediated cytotoxicity more efficiently than mAbs of IgG2b or IgG1 isotypes.

Figure 1a shows the results of a representative experiment. The curves given by serial dilutions of Abs of the three isotypes

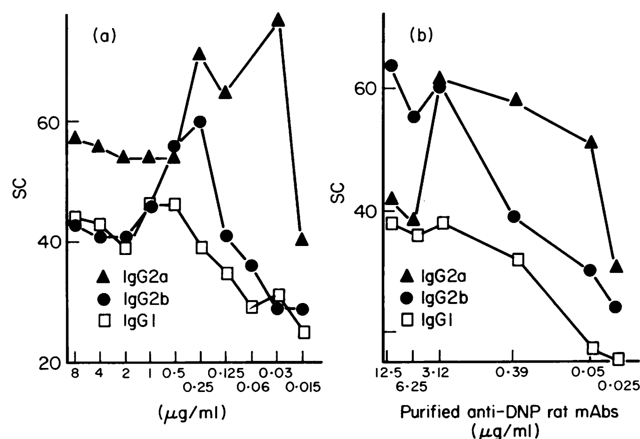

Figure 1. (a) K-cell cytotoxicity mediated by rat spleen cells. ^{51}Cr-labelled Chang-TNP were sensitized with dilutions of rat ascitis: mAbs of (▲) IgG2a (DNP-7), (●) IgG2b (DNP-57) and (□) IgG1 (DNP-2) isotypes. Polyclonal rat anti-DNP antiserum: 47% SC. NK-specific cytotoxicity value was 13% SC, BL 17%. (b) K-cell cytotoxicity mediated by rat spleen cells. ^{51}Cr-labelled Chang-TNP were sensitized with dilutions of purified rat mAbs of (▲) IgG2a (DNP-7), (●) IgG2b (DNP-57) and (□) IgG1 (DNP-2) isotypes. Figures indicate final concentrations in the assay in μg/ml. NK was 15% SC, BL 21%. Results are the mean of duplicate determinations.

are compared starting from the same concentration of Abs in ascites. IgG2a (LO-DNP-7) cytotoxicity was highest and measurable up to a 1:256·000 dilution of ascites, i.e. 0·015 μg/ml concentration in the assay. For IgG2b (LO-DNP-57) titre was 0·06 μg/ml, and for IgG1 (LO-DNP-2) 0·125 μg/ml. Differences in titres of the other mAbs, when they existed, were related to concentrations of Abs in ascites so that we found little difference in activities of the various antibodies of a given subclass.

In order to determine if the differences between IgG subclasses reported above were indeed relevant to the isotype, and to avoid the interference of possible inhibitory components in ascitis, monoclonals of each isotype were again selected and purified from ascites: LO-DNP-7 (IgG2a), LO-DNP-57 (IgG2b) and LO-DNP-2 (IgG1).

Rat effector cells were used as before. Greatest cytotoxicity was obtained when target cells were sensitized with IgG2a antibodies (Fig. 1b). IgG2b antibodies were also able to sensitize target cells so that comparable levels of K-cell-mediated killing was reached but cytotoxicity dropped off more rapidly on dilution of IgG2b antibodies. The maximum level of kill obtained with IgG1 antibodies was approximately 2/3 of that obtained with IgG2a. Respective titres were 0·025 μg/ml for IgG2a (LO-DNP-7), 0·05 μg/ml for IgG2b (LO-DNP-7) and 0·29 μg/ml for IgG21 (LO-DNP-2). This was confirmed in two other experiments. Little difference was thus observed between ascitis and purified mAbs titres, indicating that purification procedure has not altered the antibody activity.

Anti-DNP rat monoclonal of IgE isotype (Fig. 2). One rat monoclonal of IgE isotype, LO-DNP-30, was tested in our system. The cytotoxicity mediated by this reagent was found in four separate experiments (Table 1). Maximum cytotoxicity was reached at 1:2·000 and the minimum ascites dilution giving cytolysis was 1:16·000. When effectors were prepared so that the cell population was enriched in K-cell effectors, cytotoxicity was augmented (Table 3). This was the case for both IgE and IgG2a,

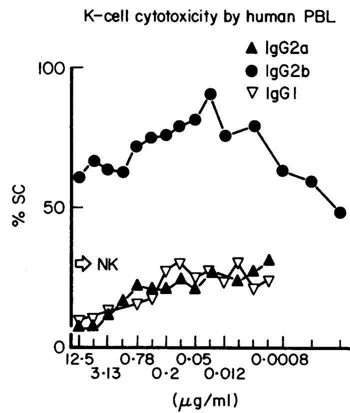

Figure 2. K-cell cytotoxicity mediated by human peripheral blood mononuclear cells. ^{51}Cr Chang-DNP were sensitized with dilutions of purified rat mAb of (●) IgG2b (DNP-57), (▲) IgG2a (DNP-7) and (▽) IgG1 (DNP-1) isotypes. Effector: target cell ratio was 20:1. The arrow gives the NK level in that experiment (30%, SC). BL was 20%. A polyclonal rabbit anti-Chang induced 80% cell lysis of Chang cells targets (NK was 10% SC). Results are the mean of duplicate determinations.

strongly suggesting that IgE-induced cytotoxicity measured here is indeed due to K cells. IgE antibody LO-DNP-30 was not tested as purified Ab as it is sensitive to pH changes occurring during purification procedure.

Anti-DNP rat monoclonals of IgA and IgM isotypes. Six IgA and two IgM anti-DNP mAbs were tested twice in three separate experiments. No cytotoxicity was recorded.

Table 3. IgG2a and IgE antibody-induced rat K-cell cytotoxicity

log 2 ascitis dilutions	IgG2a LO-DNP-7		% increase	IgE LO-DNP-30		% increase
	A	A′		A	A′	
8	NT	NT	—	39	63	62
9	46	60	30	45	65	44
10	54	51	—	48	72	50
11	NT	54	—	40	61	53
12	51	77	51	40	NT	—
13	49	75	53	48	48	—
14	50	75	50	32	32	—
15	54	77	43	37	29	—
16	52	72	38	32	28	—
17	55	62	13	34	28	—
18	45	57	27	NT	NT	—
—	20	29	45			

Specific cytotoxicity mediated by K cells from Nude rat spleens. Values shown are the mean of duplicate determinations (BL 14%).

A, whole cell population.

A′, enrichment in K cell effectors after Ficoll gradient separation and plastic adherence.

This experiment was reproduced twice with spleen cells from different donnor rats (data not shown).

For both isotypes, there is an increase in cytotoxicity when K effectors are enriched within the effector cell population.

The cytotoxicity of human K cells

When human PBL were used as effectors, no cytotoxicity was achieved by rat mAbs of the various isotypes tested, except when IgG2b mAbs were used. This was found in seven independent experiments, each Ab being assayed at least twice. Various effector: target cell ratios were used from 15:1 to 50:1. Results were not changed by increasing the number of effectors. In each experiment, PBL from a single donor were used.

Maximum cytotoxicity achieved by all of the seven IgG2b rat mAbs tested within a same experiment was 73–81% SC at a ratio of 25:1 for an antibody concentration of 0·06 µg/ml in the assay. Activity was measurable up to $2{\cdot}5\times10^{-5}$ µg/ml.

Results obtained with purified mAbs are depicted in Fig. 2. With LO-DNP-57 (IgG2b), maximum lysis of 90% SC was reached at a final concentration of $2{\cdot}5\ 10^{-2}$ µg/ml and titre was 5×10^{-5} µg/ml. DNP-7 (IgG2a) and DNP-1 (IgG1) were inactive. However, above 0·4 µg/ml, there was a killing lower than NK cytotoxicity level, indicating that anti-DNP antibodies may block the increased human NK found against hapten-modified targets compared with NK sensitivity of the same unmodified targets.

DISCUSSION

We report here direct evidence of the involvement of certain isotypes of rat immunoglobulins and not others in rat K-cell-mediated cytotoxicity. That IgG and not IgM is involved in inducing K-cell lysis in the rat agrees with an earlier report (MacLennan & Howard, 1972).

IgE-dependent K-cell cytoxocity had not been recognized previously. Although we tested only one rat mAb, positive results were obtained in each experiment, including tests with euthymic rat effector cells. We also tested an anti-DNP mouse monoclonal of IgE isotype, the MAS 039b, clone IgE 53-569 HL distributed by SERALAB. Culture supernatant of unknown concentration gave positive results at dilutions of up to 1:128. The maximum level of lysis obtained was of the same order of that given by LO-DNP-30 in the same experiment (data not shown). Such results indicate that human IgE may induce cytolysis with human K cells, although, by a direct approach, chimeric IgE with human Fc was found to be inactive with human K cell (Bruggemann *et al.*, 1987). In the rat, it is now possible to investigate further the hypothesis of a K cell IgE Fc receptor. It is indeed intriguing that the most potent IgG isotype in the rat system is IgG2a, an immunoglobulin with anaphylactic properties in the rat species. We found here that IgA Abs do not work with spenic effectors. This does not exclude the possibility that K effectors from different tissues (i.e. mucosae) may have Fc receptors for IgA. In a previous work (Chassoux & Bazin, 1982) evidence was provided to support the classification of IgE as a systemic immunoglobulin, as opposed to IgA as a mucosal immunoglobulin. The results we have with K cells strengthen this point further.

Quantitative differences were observed between the capacity of IgG2a, IgG2b and IgG1 antibodies to induce cytolysis mediated by rat K cells. This indicates either that Fc receptors on rat K cells have a different affinity for each of these isotypes, or that several types of Fc receptors for IgG exist at the K-cell membrane. Distinct receptors for IgG1/IgG2b and IgG2a have been described on rat macrophages (Boltz-Nitulescu, Bazin & Spiegelberg, 1981). This, however, was not confirmed by results

obtained by Denham, Barfoot & Jackson (1987) showing that rat macrophages only have a receptor for rat IgG2b. A modulation of the number of receptors by serum factors as immunoglobulin concentration or the blockade of receptors by immune complexes should also be considered. We found that the higher activity induced by IgG2a antibodies compared with other isotypes also applied when euthymic rat effector cells were used. Nude rats do not produce IgG2a antibodies so that Ig concentration is unlikely to be an important factor for the expression of Fc receptors for this isotype. Studies on the IgG Fc receptors of K cells will require a pure cell population or a cell line displaying K-cell activity.

In the human, inhibition experiments with aggregated myelomatous proteins had shown that all four classes of IgG were involved in K-cell lysis (MacLennan *et al.*, 1973), IgG2 and IgG4 displaying a lower affinity for K cells (Spiegelberg, Perlmann & Perlmann, 1976). By a direct approach, using chimeric antibodies (human Fc), it was shown recently that IgG1 and IgG3 only would induce human K-cell-mediated cytotoxicity (Bruggemann *et al.*, 1987). In another Ag–Ab system, when chimeric molecules with a human Fc and rat antibody function were tested for induction of human K-cell-mediated cytolysis, IgG3 was found to have only a small activity at a relatively high concentration (10 μg/ml) compared to IgG1 (Riechmann *et al.*, 1988). A heterogeneity in the level of effector function triggering of chimeric Ab molecules appears to exist. Several other systems need to be tested before a definite conclusion may be drawn on the role of isotype in human K-cell activity.

Human K cells mediated cytotoxicity efficiently with all the IgG2b mAbs tested. None of the other isotypes were able to do so. We know from the work of Christiaansen & Sears (1984) and Hale, Clark & Waldmann (1985) that rat IgG2b antibodies are able to promote antibody-dependent cytotoxicity with human effector cells. The latter authors also showed that IgG2a and IgG2c (not tested here) did not induce K-cell cytotoxicity. Here, we have extended the observations to IgG1 and IgA. Thus, we can say definitely that among rat isotypes IgG2b only induces human K cells to lyse relevant target cells. It is interesting that, in homologous situations, rat IgG2b is relatively less efficient. This is reminiscent of observations in other systems: rabbit IgG binds to human monocytes more than to rabbit macrophages, mouse IgG2a binds to mouse macrophages less than to human monocytes, indicating that relatively weak homologous binding is a function of the receptor structure (Burton, 1985). That rat IgG1 is able to fix human complement (Medgeysi *et al.*, 1978; Bazin & Lebacq, 1983) but does not bind human K cells adds to the observations indicating that a different site of immunoglobulin is involved in each lytic system: both Cγ3 (MacLennan, Connell & Gotch, 1974) and Cγ2 (Sarmay *et al.*, 1984) are necessary for K-cell-mediated lysis.

The remarkable efficiency of rat IgG2b mAbs with human effectors may provide important therapeutic applications (Hale, Clark & Waldmann, 1985). Target cells that are most susceptible to K-cell-mediated lysis are generally dividing normal or tumour cells (MacLennan, 1972; Sears & Christiaansen, 1985). Normal lymphocytes sensitized appropriately can be destroyed by K cells but SC values do not exceed 50–60% even in the case of chimeric or reshaped antibodies (Brüggemann *et al.*, 1987; Riechmann *et al.*, 1988). On the other hand, in the present study, 80–100% lysis may be obtained regularly when hapten-specific rat mAbs are used against hapten-coated tumour cells. Activated lymphocytes may also be destroyed efficiently when sensitized with an anti-HLA antibody or an antibody against differention antigen as the IL-2R (D. Chassoux and H. Bazin, unpublished data). One should note also that most Abs eliciting cytotoxicity by K cells require higher concentrations than rat antibodies, and they they are usually less efficient. Finally, whereas all rat IgG2b mAbs reported up to now are active in human K-cell cytolysis, certain mouse antibodies of the IgG2a (active) isotype are inoperant (Christiaansen & Sears, 1984) (reviewed by Chassoux *et al.*, 1988).

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