

## SPECIFIC TARGETING OF HUMAN PERIPHERAL BLOOD T CELLS BY HETEROAGGREGATES CONTAINING ANTI-T3 CROSSLINKED TO ANTI-TARGET CELL ANTIBODIES

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We have recently shown (1) that the specificities of cloned human CTL can be altered by incubating them with heteroaggregates containing anti-T3 covalently crosslinked to nominal anti-target cell antibodies. Such heteroaggregates link the target cells directly to T3 molecules on the effector cells, leading to both conjugate formation and target cell lysis. T3 is a subunit of the T cell receptor complex (2, 3), and previous experiments (4) demonstrated that a direct linkage of the target to T3 would trigger lysis. By using appropriate heteroaggregates, cloned CTL could be made to lyse almost any specified target, including allogeneic and xenogeneic tumor cells and chicken red blood cells (CRBC)<sup>1</sup> (1). Since it is unlikely that each of the CTL clones used in those experiments recognized MHC antigens on all of the target cells, it was concluded that MHC components were not involved in heteroaggregate-dependent lysis. On the other hand, a direct linkage of the target cell to the T cell receptor complex was a requirement for lysis; crosslinking target cells to other effector cell surface components did not lead to target cell lysis, even under conditions where effector/target conjugates could be demonstrated. Similar results have recently been published by Staerz et al. (5), who showed that, in murine cells, heteroaggregates that crosslink the antigen-specific portion of the T cell receptor complex (Ti) with target cells also promote target cell lysis.

In this paper, we study lysis mediated by normal human peripheral blood T cells that have been sensitized with anti-T3-containing heteroaggregates. It is known that human peripheral blood T cells will mediate lectin-dependent lysis (6); we asked whether sensitized T cells are also competent cytolytic effectors, or are instead precursors that must first differentiate and/or proliferate before mediating lysis. Here, we show that sensitized peripheral blood T cells are, in fact, potent cytolytic effectors when taken directly from the blood, but that brief (2–4 h) exposure to IL-2 can markedly enhance their cytolytic activity. Our results suggest a strategy for attacking tumor cells and other pathogenic cells *in vivo* with sensitized autologous lymphocytes.

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<sup>1</sup> *Abbreviations used in this paper:* CRBC, chicken red blood cells; LAK, lymphokine-activated killer; SPDP, *N*-succinimidyl-3-(2-pyridyl)dithiol) propionate.

## Materials and Methods

**Crosslinked Antibodies.** Anti-T3 (OKT3), anti-K<sup>k</sup> (36-7-5), and anti-DNP were purified as described (1). Anti-class I (W6/32), an anti-HLA class I framework antibody (7), was purified using a procedure like that for anti-T3. The Fab fragments of anti-T3 and anti-DNP were prepared by incubating the proteins for 4 h at 37°C with a 1:100 (wt/wt) ratio of papain/Ig in 0.1 M sodium phosphate, 0.1 M NaCl, 1 mM EDTA, 20 mM cystein, pH 8.0. The reaction was terminated by adding iodoacetamide to a final concentration of 30 mM. Analysis by SDS-PAGE indicated that the proteins had been completely digested to 50 kD fragments. They were then dialyzed against 0.1 M Tris/Cl, pH 8.5, and passed over a protein A-Sepharose column to remove Fc fragments and trace contaminants of intact IgG. Proteins were crosslinked as described (8) using the heterobifunctional cross-linking reagent, SPDP. A threefold molar excess of SPDP to protein, for both intact Ig and their Fab fragments, was used in all cases. After the crosslinking step, a small amount of iodoacetamide was added, and each sample was applied to a 1.6 × 90 cm Ultrogel AcA 22 (LKB Instruments, Inc., Gaithersburg, MD) column to remove the monomeric IgG. In most experiments, a fraction containing mostly dimers and trimers was used. However, larger aggregates were equally active and were used in some experiments. We refer, in this paper, to crosslinked antibody preparations as antibody 1 × antibody 2. The preparations used were anti-T3 × anti-K<sup>k</sup>, anti-T3 × anti-DNP, anti-T3 (Fab) × anti-DNP (Fab), and anti-class I × anti-DNP.

**Target Cells.** Four target cells were used in this paper; RDM4 and EL4 are, respectively, H-2<sup>k</sup> and H-2<sup>b</sup> murine T cell lymphomas, and M16 (9) is a human EBV-transformed lymphoblastoid cell line. CRBC were also used as targets. In some experiments, target cells were modified with TNP by incubating them with 3 mM trinitrobenzene sulfonate for 10 min at 37°C in PBS, pH 7.4. Cells were <sup>51</sup>Cr-labeled as described (10).

**Effector Cells.** Mononuclear cells were isolated from freshly drawn, heparinized venous blood from normal donors by density gradient separation over Ficoll-Hypaque (LSM; Litton Bionetics, Kensington, MD). Monocytes were depleted by passage over Sephadex G10 as described (11), yielding a lymphocyte preparation. The Leu-11<sup>+</sup> cells (including most of the NK activity) were removed from the lymphocytes by incubating them with anti-Leu-11b (Becton, Dickinson & Co., Mountain View, CA) and rabbit complement (Pel Freeze, Rogers, AK) using Becton Dickinson's suggested protocol.

In some experiments, large numbers of cells were obtained from a normal donor by leukapheresis. In those experiments, cells were cryopreserved and stored in liquid nitrogen (12) as either mononuclear cells, lymphocytes, or Leu-11-depleted lymphocytes.

**Depletion of T4<sup>+</sup> and T8<sup>+</sup> Cells.** Frozen Leu-11-depleted lymphocytes were thawed and washed. Cells (2 × 10<sup>6</sup> cells/ml) were then incubated for 30 min at room temperature with a 1:100 dilution (final concentration) of ascites produced by either OKT4 or OKT8 hybridomas (ascites were a kind gift from Dr. William Biddison). Rabbit complement was added to a 1:10 final concentration. Cells treated with OKT8 were incubated for 60 min at 37°C and washed. Cells treated with OKT4 were incubated 30 min at 37°C, washed, and treated an additional 45 min with fresh complement.

**Analyses of Cell Populations.** Cell preparations were analyzed by flow cytometry for the expression of the M1, T3, T4, T8, and Leu-11 antigens by using FITC derivatives of mAb OKM1, OKT3, OKT4 (Ortho Diagnostics, Raritan, NJ), Leu-2, and Leu-11a (Becton, Dickinson & Co.), respectively. Cells (1–3 × 10<sup>6</sup>) were pelleted and resuspended in 25 μl of FACS medium (HBSS without phenol red containing 2.5% BSA and 0.2% sodium azide) containing a saturating concentration of the appropriate FITC-antibody. After 30-min incubation at 0°C, cells were washed twice, resuspended in 1 ml of cold FACS medium, and analyzed with a FACS II cell sorter (Becton Dickinson Immunocytometry Systems, Mountain View, CA) as described (13). Control cells were prepared by incubating the cells with an irrelevant FITC-antibody of the same isotype as the test antibody.

**Incubation of Effector Cells with Lymphokines.** In an early experiment, cells were incubated with delectinated human T cell growth factor (Cellular Products, Buffalo, NY). In most experiments, however, recombinant human IL-2 (Cetus, Emeryville, VA; a gift from

Dr. Steven Rosenberg) was used. We also tested recombinant human IFN- $\gamma$  (Biogen, Cambridge, MA; a gift from Dr. Michael Lotze) for activity. In the short-term kinetic assay, 10-ml aliquots of Leu-11-depleted lymphocytes ( $10^6$  cells/ml) were incubated in 25-cm<sup>2</sup> tissue culture flasks in RPMI 1640 containing 10% FCS, 10 mM Hepes, 50  $\mu$ M 2-ME, glutamine, pyruvate, nonessential amino acids, penicillin, and streptomycin (culture medium). All flasks were incubated for 24 h in a 37°C CO<sub>2</sub> incubator, and at various intervals, recombinant IL-2 (final concentration, 10 U/ml) was added to a different flask. After 24 h, all flasks were harvested and the cells were tested for cytotoxic activity. Long-term assays for activation were done similarly, except that all cells were incubated for a total of 3 d, and IL-2 was added at 1-d intervals.

*Cytotoxicity Assays.* Cytotoxicity was measured in culture medium using 96-well, U-bottom microtiter plates (10). To each well was added varying numbers of effector cells in 100  $\mu$ l medium, followed by 20  $\mu$ l of antibody or medium and either  $10^4$  or  $5 \times 10^3$  <sup>51</sup>Cr-labeled target cells in 100  $\mu$ l medium. Plates were incubated for 4 h at 37°C in 5% CO<sub>2</sub>, 100% humidity. Supernatants were harvested with a Titertek harvesting system (Skatron, Sterling, VA) and counted in a well-type gamma counter. Triplicate determinations were made for each sample. Maximum lysis was measured by incubating target cells in 5% Triton X-100, and spontaneous release was determined by incubating the target cells in medium alone. Percent lysis was calculated as  $100 \times [(experimental\ release - spontaneous\ release)/(maximum\ release - spontaneous\ release)]$ . In some experiments, curves of percent lysis vs. E/T were analyzed by fitting them to a hyperbolic function using the MLAB program (14).

## Results

*Lysis of Target Cells by Heteroaggregate-treated Peripheral Blood T Cells.* We have previously shown (1) that cloned human CTL, when coated with anti-T3  $\times$  anti-target cell antibody would specifically lyse cells expressing epitopes recognized by the antitarget antibody. To determine whether human peripheral blood T cells could also be rendered cytotoxic for specified targets with appropriate crosslinked antibodies, mononuclear cells were incubated with <sup>51</sup>Cr-labeled RDM4 in the presence of anti-T3  $\times$  anti-K<sup>k</sup> or with <sup>51</sup>Cr-labeled TNP-RDM4 in the presence of anti-T3  $\times$  anti-DNP (Table I). In both cases, the mononuclear cells lysed the target cells when crosslinked antibody was present, but did not mediate lysis in the absence of antibody. However, mononuclear cells also mediated ADCC, as shown by target cell lysis in the presence of anti-K<sup>k</sup> (which is an IgG2a antibody). Since the crosslinked antibodies possessed Fc fragments, it was possible that tumor cell lysis in the presence of the antibody heteroaggregate represented ADCC (15) and not lysis mediated by sensitized peripheral blood T cells. To eliminate this possibility, mononuclear cells were first passed over Sephadex G10 to remove monocytes. This removed essentially all of the cells falling within the OKM1 bright peak (i.e., monocytes [13] (Table II)). As a result of monocyte depletion, ADCC activity was substantially reduced, while heteroaggregate-dependent lysis increased (Table I). An aliquot of the G10-passed cells was then treated with anti-Leu-11b and complement to remove the K, NK subset of cells. (The Leu-11 epitope is on the neutrophil and K cell Fc $\gamma$  receptor, but not on monocyte Fc $\gamma$  receptors [16].) This treatment removed >70% of the Leu-11<sup>+</sup> cells and all of the remaining ADCC activity, (Table I), leaving a population of lymphocytes that was 87% T3<sup>+</sup> (Table II), and which required the anti-T3-containing heteroaggregate to mediate lysis.

In a preliminary experiment, we found that the heteroaggregate-dependent

TABLE I  
*Heteroaggregate-dependent Lysis of RDM4 Cells by Peripheral Blood T Cells*

Exp.	Antibody	Percent lysis with various effectors			
		Mononuclear cells	Monocyte-depleted cells (lymphocytes)*	Leu-11-depleted lymphocytes <sup>‡</sup>	IL-2-activated cells <sup>§</sup>
1 <sup>†</sup>	None	-0.6	1.8	-0.3	4.6
	Anti-T3 × anti-K <sup>k</sup>	10.1	16.2	26.0	43.2
	Anti-K <sup>k</sup>	18.5	8.4	-2.3	3.9
2 <sup>†</sup>	None	-1.8	-1.0	-1.4	3.1
	Anti-T3 × anti-DNP	6.2	7.5	8.4	38.2
	Anti-K <sup>k</sup>	5.7	2.1	0.0	2.6

\* Depleted by passage over Sephadex G10.

<sup>‡</sup> Depleted by treatment of the G10-passed cells with anti-Leu-11b and complement.

<sup>§</sup> Leu-11-depleted lymphocytes were incubated overnight with 50 (Exp. 1) or 30 (Exp. 2) U/ml of recombinant IL-2.

<sup>†</sup> Tested against <sup>51</sup>Cr-labeled RDM4 at an E/T ratio of 20:1 in a 4-h assay. Anti-T3 × anti-K<sup>k</sup> and anti-K<sup>k</sup> were added to wells at 10 and 5 μg/ml final concentrations, respectively. Anti-K<sup>k</sup> is 36-7-5, a mouse IgG2a mAb.

<sup>†</sup> Same as in Exp. 1 except targets were TNP-RDM4, and anti-T3 × anti-DNP was used at 5 μg/ml. Effector cells were from a different donor than in Exp. 1.

TABLE II  
*Compositions of Human Peripheral Blood Effector Populations*

Marker	Percent positive cells using various effectors		
	Mononuclear cells	Monocyte-depleted cells (lymphocytes)	Leu-11-depleted lymphocytes
M1 (bright)	16.4	0.4	0.5
T3	66.1	84.1	87.1
T8	27.1	32.6	34.0
Leu-11 (bright)	5.1	5.6	1.6

Cells used in Exp. 1, Table I were stained for M1, T3, T8, and Leu-11 expression with FITC-labeled OKM1, OKT3, Leu-2, and Leu-11a antibodies, respectively, and analyzed by flow cytometry. Mononuclear cells, in addition to the bright peak, gave a Leu-11 dull peak that was removed by passage over Sephadex G10 and was presumably due to Fc binding.

lytic activity of T cells increased after overnight incubation with crude lymphokine-containing supernatants. The data in Table I and Fig. 1 show that lysis mediated by sensitized T cells was also markedly enhanced by overnight incubation with recombinant IL-2. IL-2 also elicited a small amount of antibody-independent lysis (Table I). This presumably arose from the contaminating Leu-11<sup>+</sup> cells remaining after treatment with anti-Leu-11b and complement (Table II). Fig. 1 shows that, unlike IL-2, recombinant human IFN-γ did not stimulate lysis mediated by heteroaggregate-treated T cells. However, in this same experiment (data not shown), IFN-γ elicited a twofold increase in ADCC activity in

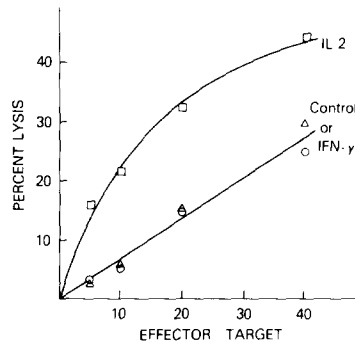


FIGURE 1. Stimulation by IL-2 of lysis mediated by heteroaggregate-treated peripheral blood T cells. Human PBL were depleted of Leu-11<sup>+</sup> cells and tested for lysis against RDM4 cells in the presence of anti-T3 × anti-K<sup>k</sup> (10 μg/ml). Cells were incubated overnight at 37°C with (Δ) medium, (□) 50 U/ml recombinant IL-2, or (○) 100 U/ml recombinant IFN-γ. In control experiments (not shown) lysis was not observed in the absence of antibodies, while ADCC (lysis in the presence of anti-K<sup>k</sup>) was detectable only after incubation with IL-2, and at E/T ratios of 40:1 (9.4%) and 20:1 (3.9%).

TABLE III  
*Specificity of Lysis by Heteroaggregate-treated Peripheral Blood T Cells*

Antibody	Percent lysis of various targets				
	RDM4	TNP-RDM4	M16	TNP-M16	EL-4
None	-2.3	4.5	2.7	3.0	3.3
Anti-T3 × anti-K <sup>k</sup>	36.3	60.3	3.5	—	—
Anti-T3 × anti-DNP	-0.9	59.6	4.1	58.1	2.3

Leu-11<sup>-</sup> lymphocytes were incubated 18 h with 100 U/ml of recombinant IL-2. Lysis was assayed in a 4-h <sup>51</sup>Cr-release assay at an E/T ratio of 20:1. —, not done.

the unfractionated mononuclear cells and in the lymphocytes before treatment with anti-Leu-11b and complement.

*Specificity of Lysis.* IL-2-activated, Leu-11<sup>-</sup> lymphocytes were rendered specifically cytotoxic for designated targets by crosslinked antibodies (Table III). Thus, effector cells incubated with anti-T3 × anti-K<sup>k</sup> lyse only targets expressing the H-2K<sup>k</sup> molecule (RDM4 and TNP-RDM4), while cells incubated with anti-T3 × anti-DNP lyse only TNP-modified target cells. As was previously shown for cloned CTL (1), heteroaggregate-coated peripheral blood T cells are able to lyse both human and murine tumor cells.

In a second experiment, heteroaggregate-treated T-cells were tested for the ability to lyse innocent bystander cells. Effector cells sensitized with anti-T3 × anti-DNP lysed TNP-RDM4, but not unmodified RDM4, as expected (Table IV). The important point was that the innocent bystander cells (<sup>51</sup>Cr-labeled RDM4) were not lysed in the presence of unlabeled TNP-RDM4.

Finally, we asked whether heteroaggregates containing anti-effector cell antibodies other than anti-T3 would stimulate lysis. Heteroaggregates containing an anti-human HLA class I framework antibody neither mediated lysis nor blocked lysis mediated by anti-T3 × anti-DNP (Table V). This, along with previous results (1) showing that cloned human CTL do not lyse targets attached to either T4 or

TABLE IV  
*Heteroaggregate-coated Human T Cells Do Not Lyse Innocent Bystander Cells*

E/T ratio	Unlabeled TNP-RDM4 added*	Percent lysis of labeled targets <sup>‡</sup>	
		TNP-RDM4	RDM4
10	—	22.0	2.2
5	—	13.2	0.3
2.5	—	8.9	-2.8
10	+	ND	0.2
5	+	ND	-0.6
2.5	+	ND	0.5

Effectors are Leu-11<sup>-</sup> lymphocytes after overnight incubation with 100 U/ml recombinant IL-2. They were incubated for 1 h at 24°C with 15 µg/ml of anti-T3 × anti-DNP, and washed before being added to target cells. E/T ratios are based upon the total number of target cells, labeled plus unlabeled.

\* Samples designated positive contain 10<sup>4</sup> unlabeled TNP-RDM4 cells per well.

<sup>‡</sup> All wells contained 10<sup>4</sup> <sup>51</sup>Cr-labeled target cells. ND, not done.

TABLE V  
*Lytic Requirement for Anti-T3 in Heteroaggregates*

Antibody*	Percent lysis of various targets <sup>‡</sup>			
	CRBC	TNP-CRBC	RDM4	TNP-RDM4
None	-1.3	1.9	-1.0	6.6
Anti-T3 × anti-DNP	-1.3	20.6	0.6	16.3
Anti-class I × anti-DNP	0.1	2.7	-1.2	7.7
Anti-T3 × anti-DNP + anti-class I × anti-DNP	—	27.4	—	14.8
Anti-T3 × anti-DNP + anti-class I	—	18.9	—	19.1

\* Heteroaggregates were added to a final concentration of 5 µg/ml. When two antibody preparations were present, each was at 5 µg/ml.

<sup>‡</sup> Leu-11<sup>-</sup> lymphocytes were used as effectors in a 4-h <sup>51</sup>Cr-release assay at an E/T ratio of 2.5:1. —, not done.

TNP groups on effector cells, strongly suggest that the target cell must bind directly to the T cell receptor complex for lysis to occur. Table V also shows that human peripheral blood T cells lyse TNP-CRBC when coated with anti-T3 × anti-DNP. This suggests that MHC components are not involved in lysis mediated by heteroaggregate-coated effector cells, since it is unlikely that CRBC express MHC antigens that could be recognized by an appreciable fraction of human CTL.

*Heteroaggregate-dependent Peripheral Blood Effector T Cells Are T8<sup>+</sup>.* Sensitized effector T cells were further characterized for the expression of T4 and T8 (Fig. 2). Leu-11-depleted lymphocytes were treated with either anti-T4 or anti-T8 and complement, and were tested for the ability to lyse TNP-RDM4 cells in the presence of anti-T3 (Fab) × anti-DNP (Fab). Fig. 2 shows that untreated lymphocytes are moderately lytic, but that IL-2 greatly increases their lytic potential. Removal of the T4<sup>+</sup> population enhances activity, both in the presence or absence

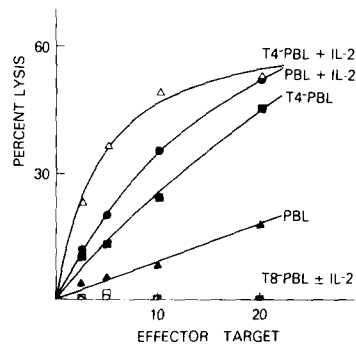


FIGURE 2. Sensitized peripheral blood effector T cells are T8<sup>+</sup>. The lytic activity of an aliquot of Leu-11<sup>-</sup> lymphocytes (PBL) was tested after overnight incubation in the presence (●) or absence (▲) of 100 U/ml recombinant IL-2. Another aliquot was treated with anti-T4 and complement and tested for lysis with (Δ) or without (■) IL-2 treatment. A final sample was treated with anti-T8 and complement, and tested with (○) or without (□) an IL-2 incubation. Targets were <sup>51</sup>Cr-labeled TNP-RDM4. Lysis was measured in a 4-h <sup>51</sup>Cr-release assay in the presence of 5 μg/ml of anti-T3 (Fab) × anti-DNP (Fab).

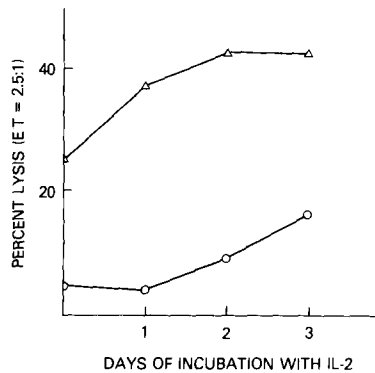


FIGURE 3. Kinetics of activation of heteroaggregate-treated peripheral blood effector T cells by IL-2. Leu-11-depleted lymphocytes were incubated in four different flasks for a total of 3 d in culture. At 1-d intervals, different flasks received 100 U/ml (final concentration) of recombinant IL-2. Flask 1 received IL-2 at the beginning of the culture period, while flask 4 received no IL-2. At the end of the 3-d culture period, cells were harvested and tested for lysis against RDM4 cells in the presence (Δ) or absence (○) of 10 μg/ml anti-T3 × anti-K<sup>k</sup>, at an E/T of 2.5:1.

of IL-2, while treatment with anti-T8 and complement totally abrogates all lytic activity. This experiment demonstrates that, within the sensitivity of the assay, all of the heteroaggregate-dependent lysis is mediated by T cells that express the T8 antigen. By using the Fab-containing heteroaggregate, we have eliminated the possibility that ADCC has contributed to the lytic process in this experiment.

*Kinetics of Activation of Sensitized T Cells by IL-2.* The kinetics of activation of heteroaggregate-coated T cells by IL-2 are shown in Fig. 3. The activation of lytic activity increased rapidly with time, being nearly complete after 1 d of incubation with IL-2. We found large variations between donors in the extents of activation of sensitized CTL in the absence of IL-2. For example, in the experiments shown in Fig. 3 and in Exp. 1 of Table I, the ability of unstimulated

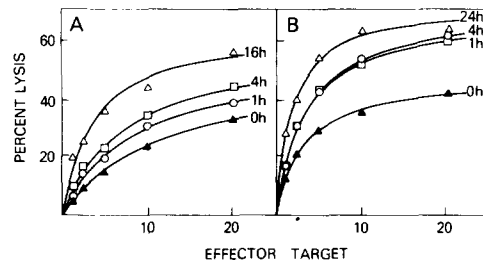


FIGURE 4. Short-term activation of sensitized peripheral blood T cells by IL-2. Leu-11<sup>-</sup> depleted lymphocytes were incubated for various periods with IL-2 as in Fig. 3, except that the total period of incubation was 24 h. Lysis was measured against <sup>51</sup>Cr-labeled TNP-RDM4 in the presence of 10 µg/ml anti-T3 × anti-K<sup>k</sup> (A) or 5 µg/ml anti-T3 (Fab) × anti-DNP (Fab) (B). Times of incubation with IL-2 are indicated. In the experiment shown, FCS was used as the serum supplement to the culture medium; similar results were obtained when human AB<sup>+</sup> serum was used.

cells to mediate lysis in the presence of crosslinked antibody was relatively high, and increased by less than twofold upon incubation with IL-2. In contrast, cells from the donors shown in Table I, Exp. 2, Fig. 1, and Fig. 2 were relatively inactive before stimulation, and showed much greater increases in lytic activity after incubation with IL-2. Antibody-independent lysis of the RDM4 tumor cells began to appear at day 2 and increased further by day 3 (Fig. 3). This presumably represents lymphokine-activated killer (LAK) activity (17, 18), and constituted less than one-eighth the total activity (in lytic units) on day 3.

Since most of the activation of sensitized T cells by IL-2 occurred within the first day of stimulation, we followed lysis at shorter intervals. Leu-11<sup>-</sup> lymphocytes were tested for the ability to lyse RDM4 cells in the presence of anti-T3 × anti-K<sup>k</sup> or TNP-RDM4 cells in the presence of anti-T3 (Fab) × anti-DNP (Fab). Experiments were done in medium containing either 10% FCS or 10% human AB<sup>+</sup> serum. Fig. 4 shows that substantial activation occurs after 1–4 h incubation with IL-2, and increases slowly thereafter. Similar results were obtained with human serum as the serum component in the culture medium (data not shown). The percent lysis vs. E/T curves can be fit within experimental error to a hyperbolic function; IL-2 causes an increase in the maximum lysis and a decrease in the E/T ratio for half-maximal lysis.

*Retention of Lytic Activity by Heteroaggregate-coated T Cells.* Because of the potential for in vivo use of sensitized effector cells, it is important to know how long after treatment with the heteroaggregate T cells can retain activity when incubated at 37°C. To test this, Leu-11<sup>-</sup> lymphocytes were activated with IL-2 and divided into two portions, one of which was incubated with anti-T3 × anti-K<sup>k</sup>, the other with medium alone. The cells were then washed and incubated for 0, 4, 8, or 24 h at 37°C and tested for lytic activity against RDM4. As seen in Table VI, the sensitized cells retained considerable activity even after 24 h. In this experiment, different target cell preparations were used to assay lytic activity at the various times of incubation. To correct for variations in lysability of the target cell preparations, as well as for variations in effector cell activity unrelated to bound heteroaggregate, untreated control effector cells were tested for lytic activity in the presence of freshly-added heteroaggregate. Table VI shows that



TABLE VI  
*etain Activity During Incubation at 37°C*

Treatment with heteroaggregate*		Percent lysis at various times of incubation (h) at 37°C <sup>‡</sup>			
Precoat <sup>§</sup>	In medium <sup>¶</sup>	0	4	8	24
-	-	2.2	3.7	4.5	6.5
-	+	33.7 (5.3)	51.4 (7.4)	42.4 (4.4)	21.9 (1.3)
+	-	33.0 (4.3)	46.6 (4.3)	48.5 (5.0)	31.4 (2.4)
+	+	32.0 (5.6)	44.3 (3.9)	49.2 (3.9)	21.4 (1.4)

\* Anti-T3 × anti-K<sup>k</sup>

<sup>‡</sup> Percent lysis of RDM4 cells by IL-2-activated, Leu-11<sup>-</sup> lymphocytes at E/T ratio of 20:1. Lytic units, defined as the initial slope of a hyperbolic fit to the E/T dose-response curve, are indicated in parentheses, where measurable. Effector cells were activated by overnight incubation with 100 U/ml recombinant IL-2.

<sup>§</sup> Effector cells were incubated 1 h at 0°C with (+) or without (-) 20 µg/ml heteroaggregate and washed before incubation at 37°C.

<sup>¶</sup> Heteroaggregate (5 µg/ml) was either present (+) or absent (-) in the lytic assay medium.

variations in lytic activity of the sensitized test cells paralleled those of the control. This suggests that variations such as the loss in activity after 24 h incubation were not due to a loss of bound heteroaggregate, but were instead due to a heteroaggregate-independent process such as the downregulation of activity following the removal of cells from IL-2.

### Discussion

Our results show that freshly purified circulating human peripheral blood T cells actively lyse tumor targets when incubated with anti-T3-containing antibody heteroaggregates, and that the specificity of lysis is dictated by the specificity of the antibody to which the anti-T3 is linked. Sensitized effectors from different donors vary in their lytic activities, but high levels of activity can be obtained from all cell samples by incubating them for a few hours with crude lymphokine-containing supernatants or with recombinant IL-2, but not with recombinant IFN- $\gamma$ . The effector cells that mediate heteroaggregate-dependent lysis express the T8 antigen, since treatment with anti-T8 plus complement removes all measurable lytic activity. However, our data do not preclude the possibility that a small subset of the cells mediating heteroaggregate-dependent lysis is T4<sup>+</sup>. In this respect, heteroaggregate-coated T cells resemble normal CTL, where the great majority are T8<sup>+</sup> (2, 19, 20). On the other hand, it is known that some T4<sup>+</sup> cells can mediate lysis (1, 21-24) and that a few cells are both T4<sup>+</sup> and T8<sup>+</sup> (25).

The T8<sup>+</sup> cells represent approximately one-third of the Leu-11<sup>-</sup> lymphocytes used in these studies. Therefore, at most, one-third of the sensitized T cells could potentially mediate target cell lysis. It is not known whether all T8<sup>+</sup> cells are cytolytic, but the activity of sensitized peripheral blood T cells approaches that of many of the cloned CTL that we have tested, suggesting that the lytic cells are not a minor subset of the T8<sup>+</sup> cells. Even before IL-2 stimulation, heteroaggregate-coated T cells from most donors exhibit appreciable lytic activity, demonstrating that circulating human T cells contain a substantial fraction of com-

petent lytic cells that do not require proliferation or differentiation to be activated. IL-2 can further activate these cells, but this occurs so rapidly that it probably involves neither cellular proliferation nor differentiation. Similar conclusions were drawn from earlier studies (6) of lectin-dependent killing of tumor targets and chicken red blood cells by human PBL. In those studies, lytic activity was also detected in freshly prepared cell samples.

Interestingly, resting splenic T cells in the mouse apparently exist in a different activation state than peripheral blood T cells in the human. In the mouse, spleen cells are unable to mediate either lectin-dependent (26) or heteroaggregate-dependent (5) lysis unless they have been preincubated for several days with IL-2 and lectin. Erard et al. (27) have recently presented strong evidence that shows that resting murine T cells must both proliferate and differentiate before becoming competent mediators of lectin-dependent lysis, in striking contrast to circulating human peripheral T cells (6).

From the results of this paper, we can begin to design a protocol for the treatment of cellular pathogens or tumor cells with sensitized T cells. For example, we envision that a reasonably specific antitumor mAb could be cross-linked with anti-T3. PBL could be obtained from a cancer patient by leukapheresis, treated briefly with IL-2, and coated with the anti-T3  $\times$  antitumor heteroaggregate (in a future communication [P. Perez, J. A. Titus, M. T. Lotze, F. Cuttitta, D. L. Longo, E. S. Groves, H. Rubin, P. J. Durda, and D. M. Segal, manuscript submitted for publication] we will show that such cells will lyse human tumor cells *in vitro*). These cells could then be reintroduced into the patient, either locally or systemically, where, if they home to the tumor mass or to secondary metastases, they would lyse the tumor cells. Such a procedure potentially would be a very efficient use of antibody. For example,  $10^8$  T cells, each with  $10^5$  T3 molecules on its surface, would bind, at saturation, a maximum of 5  $\mu$ g of crosslinked antibody. Moreover, as shown in Table VI, bound heteroaggregate would be expected to remain an effective targeting agent *in vivo* for at least 24 h (with the caveat that cells of the reticuloendothelial system might modulate the heteroaggregate from the T cell surface [28]). The heteroaggregate-coated effector T cells, because of their high abundance, should be a much stronger source of lytic activity than either NK cells or LAK cells, which, *in vitro*, require much higher E/T ratios to mediate lysis than do sensitized effector T cells (18, 29, 30).

Heteroaggregate-coated cytotoxic cells were originally used (8, 10, 31, 32) to cause ADCC effector cells to lyse specified targets. In those studies, we found that heteroaggregates containing anti-Fc $\gamma$  receptor antibodies crosslinked to anti-target cell antibodies rendered ADCC effectors specifically lytic for target cells that bound the antitarget antibody, and only heteroaggregates containing anti-Fc $\gamma$  receptor antibodies would promote lysis (8). Since two markedly different cytotoxic cells (ADCC effectors and CTL) behave similarly when coated with heteroaggregates against the appropriate receptor (Fc $\gamma$  receptor for ADCC effectors or T cell receptor complex for CTL), we suspect that most cytotoxic cells could be specifically targeted to any designated pathogen using this strategy. Moreover, anti-T3-containing heteroaggregates should, in principle, be able to target helper T cells to a lesion where they could maintain, through the produc-

tion of lymphokines, high levels of activity within the cytotoxic cell populations. Thus, by using the proper heteroaggregates and cell mixtures, it may be possible to engineer a strong and specific response against pathogenic entities which, for a variety of reasons, might normally be resistant to immunological destruction.

### Summary

Antibody heteroaggregates have been used to render human peripheral blood T cells lytic for specified targets. The heteroaggregates contain anti-T3 covalently linked to antibodies against nominal target cell antigens. Such heteroaggregates bind target cells directly to T3 molecules on effector cells and trigger target cell lysis. Freshly prepared human PBL, when coated with anti-T3-containing heteroaggregates, are lytic without further stimulation, although brief exposure to crude lymphokine-containing supernatants or recombinant IL-2, but not recombinant IFN- $\gamma$ , enhances the activity. The effector cells are T8<sup>+</sup>, and when fully stimulated, their lytic activity approaches that of some cloned CTL. When T cells are treated with heteroaggregate, washed, and incubated at 37°C in medium not containing heteroaggregate, they retain activity for at least 24 h. The results of this study suggest a strategy in which heteroaggregate-coated T cells could be used *in vivo* to mount a lytic response against pathogenic cells such as tumor cells or virus-infected cells.

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