

ACTIVATION OF Fc RECEPTOR-BEARING LYMPHOCYTES  
BY IMMUNE COMPLEXES

II. Killer Lymphocytes Mediate

Fc Ligand-induced Lymphokine Production\*

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Activation of T and B lymphocytes by binding of appropriate mitogens, antigens, or other ligands leads to lymphokine production (1-4). Wahl and co-workers (5) reported that lymphokine production by complement receptor (CR)<sup>1</sup>-bearing guinea pig lymphocytes can be initiated by binding of immune complexes. The same group mentioned similar activation of human CR-bearing cells (6). They attributed the activity to B lymphocytes. In our research, however, immune complex-induced lymphokine production (ICLP) was exclusively a property of non-B lymphocytes (7). Nylon wool nonadherent lymphocytes containing <1% membrane immunoglobulin-bearing cells produced and released leukocyte migration inhibitory factor (LIF) activity on incubation with antibody-sensitized erythrocytes (EA) or aggregated human gamma globulin (AgHGG). The capacity for ICLP was lost upon removal of lymphocytes adhering to EA monolayers, indicating that Fc receptor (FcR)-bearing cells were involved. The studies reported in this paper were designed to further characterize those cells and to determine whether their activation to LIF production is a result of interaction between their FcR and the Fc portion of IgG (Fcγ). The heterogeneity of the non-B FcR-bearing cells is well established (8-11). Some, particularly the killer (K) cells responsible for antibody-dependent cellular cytotoxicity (ADCC) (12), can be concentrated by removal of lymphocytes forming rosettes with sheep erythrocytes (RBC) (13, 14). Initial observations using this technique revealed parallel enrichment of ICLP and ADCC. This led to the study of Fcγ-induced LIF

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<sup>1</sup> *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; AgHGG, aggregated human gamma globulin; CR, complement receptor; EA, antibody-sensitized erythrocyte(s); Fcγ, Fc portion of IgG; FcR, Fcγ receptor(s); HBSS, Hanks' balanced saline solution; ICLP, immune complex-induced lymphokine production; K, killer; LIF, leukocyte migration inhibitory factor; L:T, lymphocyte: target; NK, natural killer; Percent MI, percentage of inhibition of random migration of PMN; PMN, polymorphonuclear leukocyte; RBC, erythrocyte(s).

production after more critical separation of K cells from natural killer (NK) and other FcR-bearing cells on the basis of the high-avidity binding of each to appropriate target cells (15). Using this separation technique, ICLP was found to be primarily or exclusively a function of K lymphocytes. The results indicate that the cytotoxic activity of K cells is associated with cell activation and lymphokine production. They also provide further evidence that the K cells and NK cells among normal resting lymphocytes are not identical populations, contrary to the views of others (16).

For the study of the role of Fc $\gamma$  and FcR in ICLP, we applied techniques previously used (8, 17-20) to establish the importance of Fc $\gamma$  binding to FcR in ADCC, and we followed the example of Berman and Weigel (21) in evaluating purified Fc $\gamma$  fragments as potential activators. Induction of LIF production was found to require both intact FcR and an "active" Fc $\gamma$  ligand.

The possibility is discussed that K cells have a significant noncytotoxic function in inflammatory processes by virtue of their capacity to be activated to lymphokine production by local or circulating immune complexes.

### Materials and Methods

*Fractionation of Antisera.* Rabbit antisera to chicken and sheep RBC and to P815 mouse mastocytoma cells as well as normal rabbit serum were fractionated on Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N. J.) columns to yield macroglobulin and IgG-rich fractions (22). The latter were further fractionated by batch elution from DE52 DEAE-cellulose (Whatman Inc., Clifton, N. J.) and concentrated (22). No contaminating IgM or IgG was detected in the respective purified IgG or IgM fractions by immunoelectrophoresis or double immunodiffusion in gel using goat antisera specific for rabbit  $\gamma$  or  $\mu$  chains (Dako Corp., Santa Barbara, Calif.).

F(ab')<sub>2</sub> fragments of IgG fractions of rabbit anti-chicken RBC and of normal rabbit serum were obtained by digestion with hog mucosa pepsin (Sigma Chemical Co., St. Louis, Mo.) and isolation on Sephadex G-100 (Pharmacia Fine Chemicals) (22). The preparations were tested for intact IgG by passive hemagglutination using goat anti-rabbit Fc $\gamma$  serum. No agglutination was observed.

*EA and Control RBC.* Chicken or sheep RBC were incubated with subagglutinating concentrations of the corresponding rabbit antiserum or normal rabbit serum or with IgG, IgM, or IgG F(ab')<sub>2</sub> fractions thereof. The resulting EA and control RBC were washed three times in Hanks' balanced salt solution (HBSS) and suspended in RPMI 1640 (Gibco, Grand Island Biological Co., Grand Island, N. Y.).

*Nucleated K and NK Target Cells.* P815 murine mastocytoma cells grown in RPMI 1640 containing 10% fetal calf serum were sensitized with a 10<sup>-2</sup> dilution of IgG fraction of rabbit anti-P815 serum for 30 min at 37°C for use as target cells for K cell assays. K562 human myeloid leukemia cells grown in the same manner were used as targets for NK cell assays (15).

*Soluble Fc $\gamma$  Ligands.* Deaggregated human gamma globulin was taken as the upper one-third of ultracentrifuged (40,000 g for 2 h), pooled Cohn Fraction II. AgHGG was prepared from the deaggregated preparation by heating in HBSS (1 mg/ml) at 63°C for 30 min (23). Fc $\gamma$  fragments were obtained by papain (Sigma Chemical Co.) digestion of Cohn Fraction II and isolation on DEAE cellulose and Sephadex G-100 (22). Protein concentration was measured by the method of Lowry et al. (24), and the concentration of contaminating IgG was estimated to be <0.03% of the total protein by radial immunodiffusion using goat anti-human  $\kappa$  and  $\lambda$  sera (Dako Corp.).

*Lymphocyte Suspensions.* Mononuclear cell suspensions were prepared by centrifugation on Ficoll-Hypaque (specific gravity, 1.077-1.080) (24) of diluted, heparinized fresh peripheral blood or defibrinated leukocyte concentrates recovered as by-products in the preparation of platelets for clinical use (American Red Cross Blood Services, Penn-Jersey Region, Philadelphia, Pa.). These cells (5 × 10<sup>7</sup> to 10 × 10<sup>7</sup>/ml) were washed and incubated for 45 min at 37°C in a 12-ml polypropylene syringe containing 0.5 g of scrubbed nylon fibers equilibrated with HBSS

supplemented with 10% heat-inactivated fetal calf serum. The nonadherent lymphocytes were eluted with an excess of the same HBSS. K cell-enriched lymphocytes (13, 14), were obtained by incubating nonadherent lymphocytes with 100-fold excess of washed sheep RBC for 10 min at 37°C, then centrifuging at 150 g for 5 min and leaving overnight at 4°C. The T cell-depleted fraction was removed from the interface.

All lymphocyte suspensions were prepared aseptically, washed in HBSS, and resuspended in RPMI 1640. Total cell recovery by these separation techniques was 65–95%, with 93–99% viability of the cells recovered as measured by trypan blue exclusion.

*Enumeration of Lymphocytes.* B cells were estimated by membrane immunofluorescence, T cells by rosette formation with untreated sheep RBC after overnight incubation at 4°C (26), and monocytes by cytoplasmic staining for nonspecific esterase (27). FcR-bearing cells were counted as IgG EA-rosette-forming cells (7) and as conjugates with sensitized P815 cells in the single cell agarose assay (11), and K and NK cells were enumerated in the same assay by trypan blue staining of their conjugated target cells. Percentages of the various types of cells are reported as the means and standard deviations (SD) of the results of assays performed on 3–12 lymphocyte preparations.

*Preferential Depletion and Concentration of K and NK Cells.* Nonadherent lymphocytes were mixed at a 10:1 lymphocyte:target (L:T) ratio with K562 cells fixed in 0.025% glutaraldehyde or with similarly fixed P815 cells sensitized with rabbit anti-P815 serum, or with unsensitized control P815 cells at a 30:1 ratio (15). The cell mixtures were centrifuged at 40 g, resuspended, and centrifuged on Ficoll-Hypaque. The cell populations depleted of NK and K cells, respectively, were collected at the interface, washed in HBSS, and suspended in RPMI 1640. In some experiments, the pelleted cells enriched for NK or K cells attached to fixed K562 or sensitized P815 cells were saved for culture and generation of lymphokine supernates.

*K Activity.* ADCC was measured by a microtiter modification (11) of the <sup>51</sup>Cr-release assay (28) using as targets either 10<sup>5</sup> sensitized chicken EA or 10<sup>4</sup> P815 cells sensitized with rabbit anti-P815 IgG. Activity was expressed as percent cytotoxicity at the stated L:T ratios.

*NK Activity.* These assays were performed in the same manner as in the ADCC assay except that <sup>51</sup>Cr-labeled K562 cells were used as targets.

*ICLP.* Test and control supernatants were generated by incubating the indicated numbers of lymphocytes with stated concentrations of chicken or sheep EA, antibody-sensitized P815 cells, AgHGG, or control preparations for 18 h at 37°C. The control preparations were the corresponding cells incubated with normal rabbit serum or, in the case of AgHGG, deaggregated gammaglobulin (7). LIF activity was assayed in these supernatants using a modification (7) of the indirect agarose-medium assay of Clausen (29), and results are expressed as percent migration inhibition (MI):

$$\text{percent MI} = \left[ 1 - \left( \frac{\text{test area}}{\text{control area}} \right) \right] \times 100,$$

where the test and control areas were calculated from the means of four to six replicate migration diameters. The standard errors of these pooled means were calculated using the ratio estimator (30). Because replicate values of percent MI were not normally distributed (7), the results of replicate experiments are reported as medians and ranges, and the Wilcoxon-Mann-Whitney ranking test for unpaired measurements (30) was used for statistical comparisons.

*Protease-treated Lymphocytes.* Nonadherent lymphocytes ( $5 \times 10^7$ /ml) were incubated at 37°C with 15 mg (100 U)/ml of pronase from *Streptomyces griseus* (Sigma Chemical Co.) or 2.5 mg/ml of trypsin (Gibco, Grand Island Biological Co.) in HBSS. After 30 min, an equal volume of fetal calf serum was added and the cells washed three times in HBSS containing 10% fetal calf serum and suspended in RPMI 1640. The same concentrations of trypsin abolished the ability of nonadherent lymphocytes to form rosettes with sheep RBC (32).

*Blocking Fcγ with protein A.* EA, AgHGG, and control preparations were incubated with soluble staphylococcal protein A (Pharmacia Fine Chemicals) at indicated concentrations in HBSS for 30 min at 37°C. The EA and control RBC were washed three times in HBSS before use.

## Results

*ICLP by K Cell-enriched Lymphocytes.* Nonadherent lymphocytes were enriched threefold to fourfold in K cells as well as in other Fc $\gamma$ -binding cells by depletion of sheep RBC rosette-forming cells (Table I). Such T cell-depleted lymphocyte preparations had ADCC activity at approximately one-tenth of the L:T ratios required for similar levels of <sup>51</sup>Cr-release using unfractionated nonadherent lymphocytes (Table II). These K cell-enriched lymphocytes were induced by chicken EA, AgHGG, or sensitized P815 cells to produce significant LIF activity, also at approximately one-tenth of the concentration necessary for production of similar levels of activity by unfractionated cells (Table III).

*Requirement for K Cells but Not NK Cells.* When K cells were selectively removed from nonadherent lymphocytes, <30% of the EA-rosetting cells were removed, yet ADCC and ICLP were each depleted to essentially undetectable levels (Table IV). In contrast, selective depletion of NK cells had no effect on either ICLP or ADCC, but did remove NK activity.

*ICLP by K but Not NK Cells.* High levels of LIF activity appeared in supernatants

TABLE I  
*Enrichment in FcR-bearing Cells and K Cells Associated with Removal of Sheep RBC Rosette-forming Cells from Nonadherent Lymphocytes\**

	Nonadherent lymphocytes‡	K cell-enriched lymphocytes§
EA-binding cells	15 ± 3%	46 ± 11%
Cells binding sensitized P815 cells	10 ± 1%	33 ± 3%
K cells	3.5 ± 1%	11 ± 4%

\* Means ± SD of results of three assays each.

‡ Contained <1% surface immunoglobulin-bearing cells, 69 ± 12% sheep RBC rosette-forming cells, and 0.2% esterase-positive monocytes.

§ Contained 3 ± 2% surface immunoglobulin-bearing cells, 5 ± 2% sheep RBC rosette-forming cells, and <1% esterase-positive monocytes.

TABLE II  
*Enrichment in K Cell Activity Associated with Removal of Sheep RBC Rosette-forming Cells from Nonadherent Lymphocytes*

Target cells	L:T ratio	Percent <sup>51</sup> Cr release ± SD	
		Nonadherent lymphocytes	K cell-enriched lymphocytes
Chicken EA*	20:1	51 ± 6	ND‡
	2:1	5 ± 5	46 ± 11
Sensitized P815 cells§	40:1	55 ± 10	59 ± 9
	20:1	43 ± 18	ND
	10:1	24 ± 9	59 ± 9
	5:1	12 ± 6	ND
	25:1	ND	41 ± 5
	0.6:1	ND	23 ± 6

\* Five experiments.

‡ Not done.

§ Three experiments.

TABLE III  
*Fc $\gamma$ -induced Release of LIF Activity by K Cell-enriched vs. Unfractionated Nonadherent Lymphocytes*

Fc $\gamma$ ligand/0.25 ml	Number of lymphocytes/0.25 ml	Median percent MI (range)	
		Nonadherent lymphocytes	K cell-enriched lymphocytes
10 <sup>7</sup> chicken EA	10 <sup>6</sup>	1 (-21-19)*	35 (22-62)*
	10 <sup>7</sup>	38 (21-39)*	ND‡
60 $\mu$ g AgHGG	10 <sup>6</sup>	2	45
	10 <sup>7</sup>	38	ND
25 $\mu$ g AgHGG	10 <sup>6</sup>	-4	37
	10 <sup>7</sup>	27, 31	ND
Sensitized P815 cells	10 <sup>6</sup>	ND	47 (43-53)§
	10 <sup>7</sup>	23 (22-30)§	ND

\* Five experiments.

‡ Not done.

§ Three experiments.

TABLE IV  
*Preferential Removal of K Cells but Not of NK Cells Decreases ICLP\**

Fraction of nonadherent lymphocytes	Cells used for depletion	Percent EA-rosetting cells $\pm$ SD	Percent cytotoxicity L:T 20:1		LIF activity as median percent MI (range)§
			ADCC‡	NK	
K cell-depleted	IgG-sensitized P815	9 $\pm$ 2	.8 $\pm$ 4	17 $\pm$ 8	2 (0-11)
Control	P815	13 $\pm$ 3	32 $\pm$ 10	19 $\pm$ 9	37 (35-42)
NK cell-depleted	K562	12 $\pm$ 3	42 $\pm$ 7	2 $\pm$ 4	38 (28-56)
Control	P815	14 $\pm$ 3	40 $\pm$ 8	24 $\pm$ 9	36 (31-54)

\* Results of four experiments with K cell and 3 with NK cell depletion.

‡ IgG-sensitized P815 target cells.

§ Supernatants from 10<sup>7</sup> lymphocytes incubated overnight in 0.25 ml RPMI 1640 with 50  $\mu$ g of AgHGG versus deaggregated gamma globulin.

|| Incubated with normal rabbit serum.

from small numbers of isolated K cells ( $2.5 \times 10^5/0.25$  ml) when they were incubated overnight with the antibody-sensitized glutaraldehyde-treated P815 cells used to isolate them (Table V). On the other hand, the NK cells conjugated to glutaraldehyde-treated K562 cells yielded little or no LIF activity when incubated with or without AgHGG.

*Requirement for FcR.* Nonadherent lymphocytes incubated with pronase, but not those incubated with trypsin, lost their ability to form rosettes with EA and their ability to generate LIF on incubation for 18 h with sheep EA (Table VI). When the pronase-treated, trypsin-treated, and untreated lymphocytes were incubated for 24 h, then cultured with sheep EA, no significant differences in the LIF activity of the three resulting supernatants were observed, with the pronase-treated cells having regained their FcR and their capacity for ICLP.

*Requirement for Fc $\gamma$ .* When sheep and chicken EA prepared with IgG, IgM, or F(ab)<sup>2</sup> fractions of rabbit antisera were cultured with nonadherent lymphocytes, only EA sensitized with the IgG fraction having an intact Fc region stimulated LIF

TABLE V  
*Isolated K Cells but not NK Cells Mediate ICLP\**

Conjugated cells‡	Number of conjugates/0.25 ml	Added stimulant	LIF activity of supernates as median percent MI (range)
K cells	$2.5 \times 10^5$	None	49 (47-57)§
NK cells	$5 \times 10^5$	AgHGG (25 µg)	0 (-5-11)

\* In three experiments LIF assays were performed on supernatants from overnight cultures of K cells conjugated with sensitized glutaraldehyde-treated P815 cells and of NK cells conjugated to glutaraldehyde-treated K562 cells.

‡ Cells removed in experiments reported in Table IV.

§ PMN migration in the control supernatants was not significantly different from that in RPMI 1640 alone. Deaggregated human gamma globulin and fixed P815 cells were added to control cultures. Supernatants from these produced no inhibition of migration in comparison with medium alone, indicating that neither fixed P815 cells nor conjugated K562 cells stimulated LIF production under these conditions.

TABLE VI  
*Removal of Pronase-sensitive FcR Abolishes EA-induced LIF Activity\**

Treatment of cells	Hours of preincubation‡	Percent EA-rosetting cells ± SD	Median percent MI (range)
None	0	$18 \pm 5$	44 (26-56)§
Pronase	0	$4 \pm 3$	3 (0-6)§
Trypsin	0	$16 \pm 3$	40 (23-50)
None	24	$17 \pm 3$	35 (24-37)
Pronase	24	$19 \pm 6$	21 (17-25)
Trypsin	24	$19 \pm 3$	30 (23-33)

\* In three to six experiments LIF activity was measured in supernatants of enzyme-treated and untreated nonadherent lymphocytes ( $5 \times 10^6/0.1$  ml) after incubation with equal numbers of sheep EA or control RBC.

‡ In RPMI 1640 at 37°C after enzyme-treatment and before supernatant production for LIF assay.

§  $P = 0.01$ .

production (Table VII). In addition, when sheep EA and AgHGG were preincubated with staphylococcal Protein A, there was marked inhibition of the ability of those active Fcγ ligands to induce LIF production by nonadherent lymphocytes (Table VIII).

*Activation of LIF Production with Fcγ Fragment.* Isolated human Fcγ fragments stimulated nonadherent lymphocytes to produce high levels of LIF activity (Fig. 1). The Fcγ fragments did not inhibit leukocyte migration, and they blocked EA-rosette formation by the lymphocytes.

## Discussion

Previous discussions of the function of K lymphocytes have been limited to consideration of their apparent direct cytotoxicity for antibody-coated targets (12, 28, 33). Thus, our demonstration here that at least some of the cells that mediate ADCC are those activated to release of lymphokine activity (LIF) when incubated with Fcγ

TABLE VII  
*Fc $\gamma$  Required to Stimulate Immune Complex-induced LIF Production\**

Stimulant	Sensitizing Ig fraction	Median percent MI (range)
Sheep EA	IgG‡	30 (18-45)
Sheep EA	IgM§	6 (-17-8)
Chicken EA	IgG‡	34 (28-44)
Chicken EA	F(ab') <sub>2</sub>	-1 (-5-2)

\* In four experiments nonadherent lymphocytes ( $5 \times 10^6/0.1$  ml) were incubated with equal numbers of stimulant or control cells and supernatants tested for LIF activity.

‡ Batch eluted from DEAE cellulose.

§ Macroglobulin fraction from G-200 Sephadex.

|| The passive hemagglutinating titer of this fraction was  $5 \times 10^{-6}$  using a goat antiserum to rabbit IgG, and <1:8 using a goat antiserum to Fc fragment of rabbit  $\gamma$  chain.

TABLE VIII  
*Staphylococcal Protein A Prevents EA and AgHGG from Stimulating ICLP\**

Stimulant	Protein A added per ml	Median percent MI (range)
Sheep EA	0	25 (20-43)‡
Sheep EA	10 $\mu$ g	8 (0-15)‡
AgHGG	0	31 (29-32)
AgHGG	50 $\mu$ g	7 (4-15)

\* In three to four experiments nonadherent lymphocytes ( $10^7/0.25$  ml) were incubated with equal numbers of sheep EA or control RBC or with 250  $\mu$ g/ml of AgHGG or deaggregated human gamma globulin with or without preincubation with protein A and the LIF activity of the supernatants assayed.

‡  $P < 0.05$ .

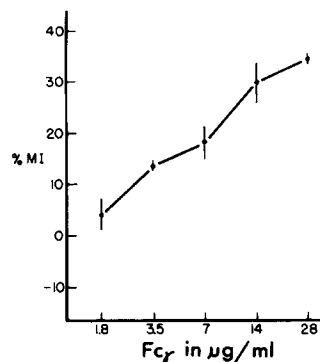


FIG. 1. Stimulation of nonadherent lymphocytes to produce LIF-active supernatants by isolated Fc $\gamma$  fragments. In a single experiment, nonadherent lymphocytes ( $10^7/0.25$  ml) were incubated with increasing concentrations of an Fc $\gamma$  fragment preparation and the supernatants tested in triplicate for LIF activity. The control supernatants were prepared by incubating the lymphocytes in RPMI 1640, then adding the appropriate concentrations of Fc $\gamma$  fragments 10 min prior to harvesting. Each point represents the percent MI calculated from the mean test and control diameters for the triplicate assays. The bars indicate the standard errors of the pooled means.

ligands assumes special significance. Immune complex-induced release of LIF activity is probably associated with actual synthesis of this lymphokine because agents that block RNA and protein synthesis inhibit its production (7). If immune complexes can induce K cells to produce lymphokines in a manner quantitatively similar to antigen-induced lymphokine production by T cells, then they might have a role in inflammatory processes *in vivo* that is far more important than their cytotoxicity for antibody-coated cells. Thus, the quantitative aspects of the results reported here deserve further analysis.

Previously it was shown (12, 34) by essentially complete depletion of activity on EA monolayers that, like K cells, the human lymphocytes responsible for ICLP bear FcR (7). In the first group of experiments reported here, lymphocyte populations enriched threefold to fourfold in K cells and other FcR-bearing lymphocytes were found to exhibit markedly increased ICLP (Tables I and III). Both the Fc ligand-induced LIF production and the ADCC activity of those K cell-enriched lymphocytes were almost equal to or greater than those found with 10-fold more unfractionated nonadherent lymphocytes (Tables II and III). The quantitative significance of this parallel increase in activity remained in doubt, however, because of the limitations and variability of both the ADCC and LIF assays, particularly because the inactive cells in less purified preparations might be expected to interfere with cell contact in the ADCC assay, to absorb or inactivate LIF (7), or to exert suppressor or stimulatory influences on the ADCC (35) or ICLP functions of the active cells. Nevertheless, the apparent parallel concentration of ADCC activity and ICLP suggested that the effector cells of these two functions might belong to the same subpopulation of FcR-bearing cells. Therefore, another depletion and enrichment procedure was used to analyze the subpopulation of FcR-bearing cells responsible for ICLP.

The preferential depletion of K or NK cells can be achieved using differences between the binding capacities of K and NK cells for their respective target cells (15). Previously, those differences were used to demonstrate that K cells and NK cells are separate subsets of FcR-bearing lymphocytes, which in turn are distinct from one or more additional subsets lacking both functions. In this investigation, we observed that the removal of the subset of FcR-bearing lymphocytes that mediates ADCC also removed cells active in ICLP (Table IV) even though >70% of FcR-bearing cells remained in the K cell-depleted lymphocyte population. In contrast, the selective removal of NK cells did not affect LIF production.

Positive selection experiments indicated that K cells are not only necessary for ICLP but that they, or a much smaller population of very closely associated cells, actually secrete the lymphokine (Table V). When cultured with the sensitized targets to which they were conjugated, the isolated K cells yielded supernatants with greater activity than the activity produced by 40 times as many nonadherent lymphocytes. On the other hand, isolated NK cells produced no measurable LIF activity when cultured with AgHGG and their conjugated target cells. In these experiments, the ICLP was clearly due to some or all of the K cells because the only difference between the test and control supernatants was the presence of lymphocytes coupled to sensitized, fixed P815 cells. Previously, it was shown that at the L:T ratios used for isolation >90% of such conjugates, which involve 2-4% of the nonadherent lymphocytes, are cytotoxic for unfixed antibody-sensitized P815 cells (15). It is, of course, possible that the LIF activity was produced by only a small subfraction of those cells.



The production of supernatants with maximum LIF activity by  $10^6$  isolated K cells per ml is comparable on a cell-to-cell basis with the production of supernatants with similar LIF activity by T cells stimulated with mitogens (29, 36) and by larger numbers of lymphocytes from highly sensitized persons incubated with optimum concentrations of antigens (37, 38). Thus, it appears that the amount of LIF activity released by K cells on stimulation by Fc $\gamma$  ligands is of the same order of magnitude as that released by similar numbers of stimulated T lymphocytes. Because the number of T lymphocytes sensitized to a given antigen would rarely be expected to reach the 2-4% of circulating lymphocytes that might respond to immune complexes, ICLP has the potential for producing inflammatory effects equal to or greater than those presumably produced by antigen-stimulated T cells.

The elimination of ICLP by depletion of K cells indicates that such cells are required but it does not address the question of whether the FcR are involved in cell activation for lymphokine production. This was investigated by testing for ICLP after enzymatic stripping of FcR from the lymphocytes (Table VI). It was shown that the removal of pronase-sensitive receptors but not of trypsin-sensitive surface proteins from lymphocytes reduced their ability to form rosettes with sheep EA and to mediate ICLP. Both were regenerated by culturing the pronase-treated cells for 24 h at 37°C. The same phenomenon has been noted in the case of ADCC (17). Although pronase is a nondiscriminatory protease that might have removed other surface membrane molecules, these data suggest that the FcR are required for immune-complex-induced LIF activity. However, they do not indicate whether Fc binding is necessary.

The involvement of Fc $\gamma$  in ICLP was confirmed by the observations that immune-complex-induced LIF production occurred only in the presence of particulate complexes with intact Fc region of IgG antibody (Table VII) and that preincubation of the complexes with the Fc $\gamma$ -binding agent, staphylococcal protein A, blocked much of the stimulating activity of those complexes (Table VIII). These data indicate that it is interaction of Fc $\gamma$  with FcR that activates K cells to produce LIF activity, but they provide no evidence regarding the possible need for binding at other sites of the immunoglobulin molecule.

Evidence that the binding of the FcR with Fc of IgG is sufficient for activation of lymphokine production without involvement of other portions of the IgG molecule comes from the experiments with isolated Fc $\gamma$  fragments. The latter stimulated nonadherent lymphocytes to produce LIF activity at protein concentrations about eightfold lower than the concentrations previously found to be required for crude AgHGG preparations (7) (Fig. 1). It is possible, however, that enzymatic degradation of IgG with papain or further degradation by mononuclear phagocytes in culture (39) exposes new sites on the Fc $\gamma$  fragment with affinity for cell membrane receptors other than the FcR that recognize IgG immune complexes.

As shown in the previous paper (7), deaggregated human gamma globulin, that is monomeric native IgG, does not induce lymphocytes to release measurable LIF. This is as expected because the avidity of the binding of monomeric IgG to the FcR of undefined lymphocytoid cells is much lower than that of aggregated or complexed IgG, and aggregates and complexes are much more effective in blocking ADCC than is native IgG (19, 40). The exact associative or conformational changes that occur in the Fc portion of IgG molecules on binding to antigen or on intermolecular aggre-

gation that make them bind more avidly to most cellular FcR and to Clq are not known (41, 42), nor is it known whether the same changes occur with enzyme excision of Fc $\gamma$  fragments. Nevertheless, it is useful to consider all altered Fc $\gamma$  ligands as active Fc to distinguish them from the Fc of native monomeric IgG, even though activation might involve only cross-linkage of FcR due to the macromolecular structure of complexes and aggregates.

The ability of isolated Fc $\gamma$  fragments to stimulate ICLP suggests the possibility that cross-linkage of the FcR is not required for cell activation by active Fc $\gamma$ . This speculation derives from the fact that the Fc $\gamma$  fragment does not readily aggregate even when heated (43) and therefore might have been presented to the lymphocyte FcR in monomeric form in our experiments. This would not preclude, however, the possibility that monomeric Fc $\gamma$  might aggregate locally on the cell membrane, as occurs with other monomeric ligands and cells (44). Such aggregation and resulting redistribution might trigger ICLP in the same manner as postulated for ADCC (19). The additional possibility that Fc $\gamma$  fragments activate K lymphocytes only after predigestion by mononuclear phagocytes, as in the case of activation of mouse B cells (39), has not yet been investigated.

In the experiment described in Table V, the stimulus to produce LIF was identical to the target cell in ADCC assays (except for glutaraldehyde fixation). In other words, it is likely that whenever a K cell exerts its cytotoxic activity on a target cell, it is simultaneously activated to release lymphokines. This is the first clear demonstration of cell activation in association with ADCC. It raises again the possibility that ADCC is itself the result of cytotoxic lymphokines produced by activated K cells (45, 46). It appears, however, that not all forms of lymphocytotoxicity are associated with secretion of lymphokines because isolated NK cells did not release detectable LIF activity when incubated with their conjugated target cells (c.f. last footnote to Table V).

Until recently, it was widely held that the K and NK activities lay within the same population (16). Evidence has been accumulating, however, that in unstimulated normal lymphocyte populations cells with K cell activity can be almost quantitatively separated from those with NK activity (15, 47, 48). The data presented in this paper document additional distinct functional differences between the FcR-bearing cells that mediate ADCC and those mediating NK activity; K cells are directly activated by immune complexes via their FcR, whereas NK cells are not activated to produce lymphokines by immune complexes. The functional differences between cells that have seemingly similar surface membrane receptors might be related to different receptors or due to differences in density of similar receptors per unit area of cell membrane. Possibly a certain number of active Fc $\gamma$  molecules must be bound or a certain concentration attained to initiate cell activation.

Because the direct inhibitory effects of immune complexes on the migration of mononuclear phagocytes (36) makes assay of macrophage-active lymphokines in ICLP more difficult, our studies to date have been limited to the PMN-active lymphokines, LIF, and chemotactic factor. It is likely that other physiologically important lymphokines are also produced in ICLP. As mentioned above, before these experiments, the primary function attributed to K cells was cytolysis of antibody-coated targets, and this has been implicated in a variety of processes *in vivo* (33, 49). The ability of K

cells to produce soluble mediators in ICLP might be the bridge between antibody-mediated effector mechanisms and cell-mediated inflammatory responses.

### Summary

Cells that participate in immune complex-induced production of leukocyte migration inhibitory factor (LIF) activity can be concentrated in a population making up 2-4% of human peripheral blood lymphocytes in which >90% of the cells are active in a single cell antibody-dependent cellular cytotoxicity assay. When so concentrated, such killer (K) cell preparations are as efficient in producing LIF activity as mitogen-activated T lymphocytes. Other Fc receptor (FcR)-bearing lymphocytes, including natural killer (NK) cells, do not produce measurable LIF activity when incubated with immune complexes (additional evidence that the K and NK cells among unstimulated human lymphocytes represent distinct populations). Binding of Fc ligands to the FcR of the appropriate lymphocytes, possibly without need for exogenous receptor bridging, is the only requirement for their activation to immune complex-induced lymphokine production (ICLP). It is probable that ICLP by K cells plays a role in antibody-mediated effector functions in vivo.

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