# Target Cell Destruction In Vitro by Concanavalin A–Stimulated Lymphoid Cells

Howard J. Schwartz, MD and Frank Wilson

**Guinea pig lymphoid cells obtained from lymph nodes and spleens of nonimmune animals are cytotoxic for mouse L-cell fibroblasts on incubation with concanavalin A. This effect is blocked by methyl-α-D-mannoside. The results indicate that this effect may be due to the release of a lymphotoxin by the concanavalin A–stimulated lymphocytes. (Amer J Path 64:295–304, 1971)**

TARGET CELL DESTRUCTION as a correlate of *in vitro* delayed hypersensitivity has been under intensive study for several years. The phenomenon appears to be the result of a two-step process mediated by lymphocytes in which adherence of lymphocytes to target cells is followed by the release of a cytotoxin and target cell death.[1–3] Although this is a specific immune event (*ie*, immune lymphocytes are stimulated to this activity only by specific antigen),[1] phytohemagglutinin apparently can induce similar activity by "nonspecific" means.[1,2,4]

Recent reports indicate that concanavalin A has profound effects on delayed hypersensitivity. It stimulates lymphocytes to blast cell transformation,[5] blocks the expression of tuberculin hypersensitivity in sensitized guinea pigs,[6] prolongs transplant survival in mice,[7] and stimulates the production of skin-reactive factor [8,9] and migration-inhibitory factor [10] by nonimmune lymphocytes. It thus seemed of interest to study the effect of concanavalin A–stimulated lymphocytes on mouse L-cell fibroblast monolayers maintained in tissue culture.

## Materials and Methods

### Animals

Randomly bred female albino guinea pigs were used throughout these experiments.

From the Department of Medicine, Case Western Reserve University School of Medicine and University Hospitals, Cleveland, Ohio.

Supported by grant T-512 from the American Cancer Society and in part by general research support grant FR05410 to Case Western Reserve University from the National Institutes of Health, and reseach grant AM01005 from the National Institutes of Health, US Public Health Service.

Accepted for publication April 5, 1971.

Address for reprint requests: Dr. Howard J. Schwartz, Department of Medicine, Case Western Reserve University School of Medicine and University Hospitals, University Circle, Cleveland, Ohio 44106.

### Reagents

Concanavalin A and methyl-α-D-mannoside were generously supplied by Dr. Myron A. Leon. Minimum essential medium, fetal calf serum, Hanks' balanced salt solution and tissue culture medium 199 were obtained from Grand Island Biological Company. Normal guinea pig serum was obtained from Hyland Laboratories. Complete medium for maintenance of L-cells was prepared by adding 45.6 ml tryptose phosphate broth and 114 ml fetal calf serum (heat-inactivated) to each 1000 ml of Eagle's minimal essential medium with Earle's salts. Prior to use, 200 units/ml penicillin, 40 μg/ml streptomycin and 1 ml *l*-glutamine (200 mM) (100×) were added to the medium.

Saline A was prepared by dissolving 8 g NaCl, 0.4 g KCl, 1.0 g dextrose and 0.35 g $NaHCO_3$ in 990 ml demineralized water. To this were added 10 ml of phenol red stock solution (2 g/100 ml demineralized water), 200 units/ml penicillin, 40 μg/ml streptomycin. Trypsin–versene solution was prepared by mixing 4 parts 0.01% versene in phosphate-buffered saline (without calcium or magnesium) with 1 part 0.25% trypsin in saline A. The 10% fetal calf serum (in Eagle's minimal essential medium with Earle's salts), saline A and trypsin–versene were filtered through a 0.2 porosity selas filter after preparation as described.

### Preparation of Lymphoid Cells

After the animals were exsanguinated by cardiac puncture, inguinal, popliteal and axillary lymph nodes, and spleens were removed, and added directly to chilled Hanks' balanced salt solution in a sterile Petri dish. The cells were gently teased out with mouse-toothed forceps and filtered twice through sterile glass wool. The cells were then washed with Hanks' balanced salt solution, centrifuged twice at 1000 RPM for 10 minutes, and finally suspended in tissue culture medium 199 containing 20% normal guinea pig serum, 200 units/ml penicillin and 40 μg/ml streptomycin for a 24-hour incubation at 37 C. After this period the cells were centrifuged, washed twice in Hanks' balanced salt solution and resuspended in 10% fetal calf serum for use in cytotoxicity assays. Cell viability (as assessed by exclusion of trypan blue) was routinely greater than 90%, and the cell preparations were 95% or more lymphocytes.

### Maintenance of Fibroblasts

Mouse L-cell fibroblast monolayers (kindly donated by Dr. Stephen Toy) were subcultured thrice weekly for maintenance of stock lines or for assay. All medium was aspirated and the monolayer washed twice with saline A. Trypsin–versene solution was then added to the flask for 10 seconds and removed by aspiration. The flasks were allowed to sit for 10–15 minutes or until the sheet of cells was freed from the flask. Cells were suspended in 10% fetal calf serum and transferred to stock flasks containing 25 ml of 10% fetal calf serum, or to 30-ml tissue culture flasks containing 4 ml of medium.

### Cytotoxicity Assay

An amount of 5–8 × $10^5$ L-cells was added to 4 ml of medium in 30-ml plastic tissue culture flasks. One day later, concanavalin A (10 μg/ml final concentration) was added to the flasks. Then, 1 × $10^7$ lymphocytes were added to each flask (control or experimental) and the cultures incubated for an additional 48 hours. The medium was aspirated off the monolayer and the cells washed twice in cold saline A and once in saline A at 37 C. The remaining attached cells were removed by incubating them at 37 C with 1.5 ml trypsin–versene. One milliliter of saline A

was added to each flask and the cells suspended evenly by gentle pipetting. One milliliter was added to 19 ml of normal saline and counted in a model B Coulter counter, thresholds, 30 to ∞; 1/amplification, ½; and 1/aperture current, 0.354. Duplicate counts were made on each culture; and the values obtained for duplicate cultures were averaged.

### Plaque Assay

An amount of 5–8 × $10^5$ L-cells was added to 4 ml of media in 30-ml plastic tissue culture flasks and cultured for 3 days at 37 C. The cells were washed twice in saline A, 4 ml of fresh 10% fetal calf serum was added, and concanavalin A (with or without methyl-α-D-mannoside, an inhibitor of the action of concanavalin A[5]) was added to the flasks. One drop of lymphocytes in 10% fetal calf serum (approximately 1.6 × $10^7$ cells) was added to the center of the tissue culture flask. Cells were incubated for 48 hours, and stained with neutral red or tetrachrome stains.

### Histologic Studies

Acid-washed 22 × 22-mm glass coverslips were placed in 35 × 10-mm sterile glass Petri dishes. Three milliliters of fibroblasts (1 × $10^5$ cells/ml) in 10% fetal calf serum were added to the dish. Twenty-four hours later, the appropriate stimulating material and 2–8 × $10^6$ lymphocytes were added. At 48 hours, the coverslips were removed, air-dried, stained for 2 minutes with tetrachrome, 3 minutes with tetrachrome buffer, and washed with water. The coverslips were air-dried and permanent slides made with permount.

## Results

When lymphoid cells from normal guinea pigs were added to L-cell fibroblast monolayers in the presence of concanavalin A, an area of clearing was seen after 24 hours (Fig 1). This effect did not occur when either concanavalin A or lymphocytes were added alone. Microscopically, one could see clustering of lymphocytes around the fibroblasts, with apparent adherence of many lymphocytes to the fibroblast cell surface (Fig 2). This effect of concanavalin A–lymphocyte interaction was prevented by the concurrent addition of 0.1 M methyl-α-D-mannoside to the system. Fibroblasts outside of the plaque area appeared normal.

The cytotoxic effect of concanavalin A–stimulated lymphocytes could be measured by counting the residual living fibroblasts that remained attached to the tissue culture flask after 48 hours of incubation. Detached cells did not exclude trypan blue dye, and therefore were judged to be dead. Concanavalin A–lymphoid cell mixtures cultured for 48 hours in tissue culture flasks containing fibroblast monolayers were cytotoxic, with fibroblast survival ranging from 19 to 62%, with a mean survival of 43.6%. These cytotoxic effects were observed at a "final" lymphocyte/target cell ratio of 5:1 (calculated as

Table 1—The Effect on L-Cell Fibroblast Survival of Their Concurrent Incubation with Concanavalin A–Lymphoid Cell Suspensions

| Experiment No. | Surviving fibroblasts ($\times 10^5$) at 48 hours in the presence of: Lymphoid cells* (a) | Lymphoid cells + Con A† (b) | Con A† | Lymphoid cells + Con A + MAM‡ | Survival at 48 hours $\left[\frac{b}{a} \times 100\right]$ (%) |
|---|---|---|---|---|---|
| 1 | 25.2 | 11.7 | 24.9 | 26.2 | 46.4 |
| 2 | 32.9 | 20.4 | 28.5 | 32.3 | 62.0 |
| 3 | 20.5 | 10.0 | 15.1 | 21.6 | 48.7 |
| 4 | 16.8 | 9.2 | 13.9 | 20.6 | 54.7 |
| 5 | 19.5 | 6.0 | 15.7 | 19.5 | 30.7 |
| 6 | 21.3 | 4.2 | 17.6 | 21.1 | 19.3 |
| Mean | 22.7 | 10.2 | 19.3 | 23.6 | 43.6 |

* $1 \times 10^7$ lymphocytes added in 0.5 ml volume.
† Con A = concanavalin A, 10 μg/ml final concentration.
‡ MAM = methyl-α-D-mannoside, 0.1 M final concentration.

the number of lymphocytes added/number of fibroblasts at the end of the experiment) (Table 1).

In an effort to determine whether this cytotoxicity was due to the elaboration of a lymphotoxin, several experiments were carried out in which unconcentrated supernatants from lymphoid cells cultured in the presence of concanavalin A (20 μg/ml final concentration) were added to fibroblast monolayers. In several experiments, cytotoxicity was observed (Table 2). While this effect was not ob-

Table 2—Cytotoxicity Obtained by Adding Supernatant Fluid from Lymphoid Cell–Concanavalin A Cultures to Mouse L-cell Fibroblasts*

| Source of supernatant fluid | L-cell death (%) |
|---|---|
| Con A† + lymphoid cells | 15 |
| Lymphoid cells alone | 0.5 |
| Con A† alone | 0.8 |
| Con A† + lymphoid cells | 12 |
| Lymphoid cells alone | 1 |
| Con A† alone | 3 |
| Lymphoid cells + Con A + MAM‡ | 0 |

* $1 \times 10^7$ lymphoid cells/ml incubated with concanavalin A, 20 μg/ml final concentration, for 24 hours.
† Con A = concanavalin A.
‡ Methyl-α-D-mannoside added to culture system with concanavalin A, at final concentration of 0.1 M.

tained in all cases, the data suggest that a cytotoxin released by concanavalin A–stimulated lymphocytes is responsible for target cell death.

Allogeneic inhibition apparently was not involved in these experiments, since the addition of suspensions of guinea pig lymphoid cells alone did not cause any discernible fibroblast cytotoxicity.

## Discussion

The recent development of a variety of *in vitro* models of delayed hypersensitivity has led to interesting new insights into the pathogenesis of this type of immune reaction. While it is quite clear that remarkable immunochemical specificity [11,12] is exhibited in delayed hypersensitivity, a variety of nonspecific mediators are released after the reaction of immune lymphocytes with antigen, including lymphotoxin, migration-inhibitory factor, blastogenic factor, skin-reactive factor, etc.[13] The nature, discreteness and identity of these mediator molecules remain unclear, as does the nature of the binding site on the lymphocyte cell membrane.

Concanavalin A is a homogeneous protein that combines with certain carbohydrate residues on a variety of proteins and cell surfaces.[5,14,15] It is also a lymphocyte stimulant, inducing blast cell transformation,[5] and the production (or release) of skin-reactive factor and migration-inhibitory factor.[8–10] In this paper, we have demonstrated that it also induces lymphocyte cytotoxicity for mouse L-cell fibroblasts.

In demonstrating this ability of concanavalin A to induce lymphocyte cytotoxicity, we have followed the established procedures of Ruddle and Waksman.[16,17] Our results in a nonimmune system with concanavalin A compare favorably with the results they obtained in an immune system with specific antigen.

Perlmann and Holm [18] have recently reviewed the subject of cytotoxicity and indicate certain drawbacks in the currently available assay systems. They point out that the technic of cell counting, such as used here, may be influenced by the presence of macrophages in the lymphoid cell population. Release of intracellular enzymes from such cells might cause cell detachment that would be spuriously attributable to lymphotoxin. We feel that this possibility, although real, does not explain our results. Our lymphoid cell preparations were routinely composed of 95% or more lymphocytes, and the ratio of lymphocytes added to target cells was only 5:1 (at the conclusion of the experiment). Hence macrophages, if present, represented only a

small fraction of the cell population and seem unlikely to have produced such clear results. Secondly, the detached cells failed to exclude trypan blue, and thus met a standard criteria for nonviability. Finally, the results of the plaque assays (Fig 1) agree with the cell counting data in indicating a cytotoxic effect of concanavalin A–stimulated lymphocytes. This ability to stimulate nonspecifically the production of several of the mediators of delayed hypersensitivity may be of great utility in the large-scale preparation and separation of the molecular materials responsible for these effects.

It is important to note that this report is in contrast with that of Perlmann *et al*,[19] who have described the inhibition of phytohemagglutinin-induced cytotoxicity for chicken erythrocytes by pretreating human lymphocytes with concanavalin A. This discrepancy may be explained by differences at the level of either the effector cell or target cell. In this report, preparations of guinea pig lymphoid cells were utilized, whereas Perlmann *et al* used preparations of human lymphocytes. It is possible that there are significant variations in the susceptibility of lymphocytes from different species to activation by concanavalin A.

On the other hand, Williams and Granger have reported that not all tissue culture cell lines are uniformly sensitive to lymphotoxin-induced cytolysis.[20] Apparently, the mouse L-cell fibroblast is much more sensitive than a variety of other cells, and the use of this cell line may account for our noting cytotoxicity after the interaction of concanavalin A–stimulated lymphocytes with the L-cells. It is possible that chicken erythrocytes differ from mouse fibroblasts in some basic structural or metabolic characteristic that accounts for different vulnerabilities to cytolysis. Thus, while concanavalin A–induced lymphotoxin did not result in erythrocytolysis,[19] it may have blocked lymphocyte receptor sites, or somehow depleted lymphocyte stores of lymphotoxin, and thus prevented the action of phytohemagglutinin in that system. It is to be hoped that the resolution of this difference, as well as the general mode of action of concanavalin A, will lead to further insights into the mechanisms of expressing cellular immunity.

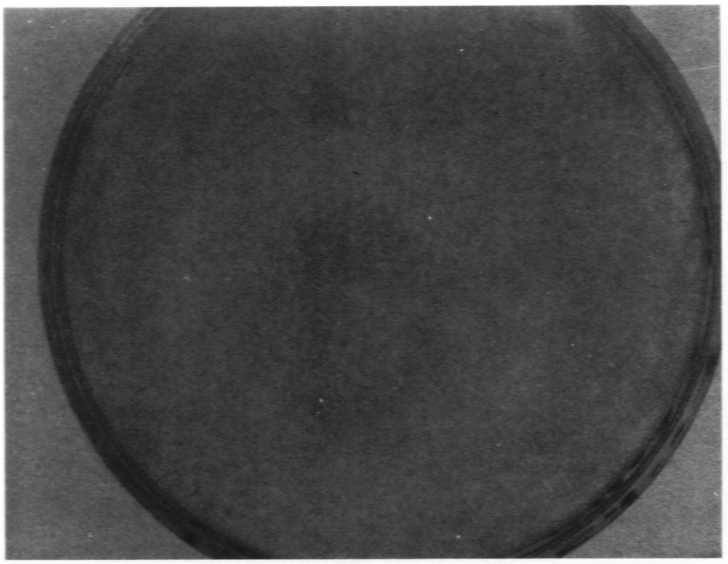
## References

1. Rosenau W: Target cell destruction. Fed Proc 27:34–38, 1968
2. Ruddle NH, Waksman BH: Cytotoxicity mediated by soluble antigen and lymphocytes in delayed hypersensitivity. III. Analysis of mechanism. J Exp Med 128:1267–1279, 1968
3. Granger GA, Williams TW: Lymphocyte cytotoxicity *in vitro:* activation and release of a cytotoxic factor. Nature 218:1253–1254, 1968

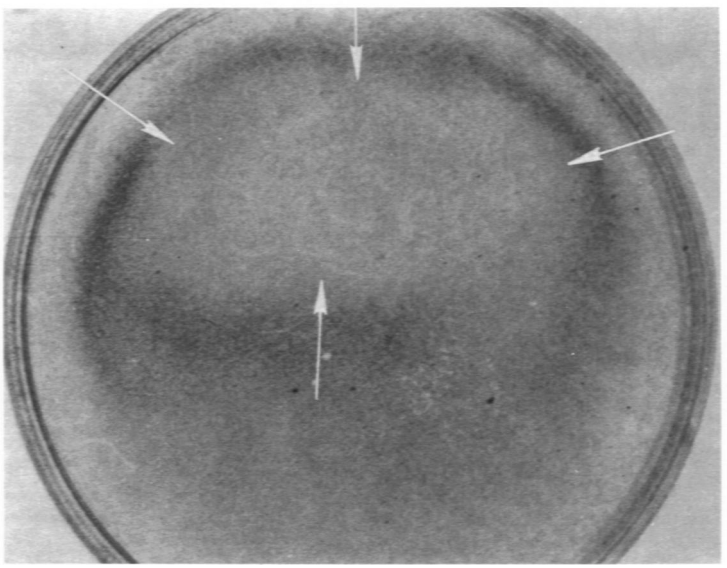
4. Holm G, Perlmann P, Werner B: Phytohemagglutinin-induced cytotoxic action of normal lymphoid cells on cells in tissue culture. Nature 203:841–843, 1964
5. Powell AE, Leon MA: The reversible interaction of human lymphocytes with the mitogen concanavalin A. Exp Cell Res (in press)
6. Leon MA, Schwartz HJ: Inhibition of delayed hypersensitivity to tuberculin by concanavalin A. Proc Soc Exp Biol Med 131:735–736, 1969
7. Markowitz H, Person DA, Gitnick GL, Ritts RH Jr: Immunosuppressive activity of concanavalin A. Science 163:476, 1969
8. Schwartz HJ, Leon MA, Pelley RP: Concanavalin A induced release of skin-reactive factor from lymphoid cells. J Immun 104:265–268, 1970
9. Pick E, Krejci J, Turk JL: Release of skin reactive factor from guinea pig lymphocytes by mitogens. Nature 225:236–238, 1970
10. Schwartz HJ, Pelley RP, Leon MA: Release of migration inhibitory factor from non-immune lymphoid cells by concanavalin A. Fed Proc 29:360, 1970, abstr
11. Schlossman SF, Levine H: Desensitization to delayed hypersensitivity reactions: with special reference to the requirement for an immunogenic molecule. J Immun 99:111–114, 1967
12. Leskowitz S: Some relations between the specificity of antibody and delayed hypersensitivity. Immunochemistry 4:91–94, 1967
13. Lawrence HS, Landy M, editors: Mediators of Cellular Immunity. New York, Academic Press, Inc, 1969
14. Harris H, Robson EB: Precipitin reactions between extracts of seeds of Canavalia ensiformis (Jack Bean) and normal and pathological serum proteins. Vox Sang 8:348–355, 1963
15. Leon MA: Concanavalin A reaction with human normal immunoglobulin G and myeloma immunoglobulins. Science 158:1325–1326, 1964
16. Ruddle NH, Waksman BH: Cytotoxic effect of lymphocyte-antigen interaction in delayed hypersensitivity. Science 157:1060–1062, 1967
17. Ruddle NH, Waksman BH: Cytotoxicity mediated by soluble antigen and lymphocytes in delayed hypersensitivity. I. Characterization of the phenomenon. J Exp Med 1237–1254, 1968
18. Perlmann P, Holm G: Cytotoxic effects of lymphoid cells *in vitro*. Advances Immun 11:117–193, 1969
19. Perlmann P, Nilsson H, Leon MA: Inhibition of cytotoxicity of lymphocytes by concanavalin A *in vitro*. Science 168:1112–1115, 1970
20. Williams TW, Granger GA: Comparisons of the sensitivities of various cell lines to human lymphotoxin. Fed Proc 29:306, 1970, abstr

Mr. Wilson is currently a medical student, Ohio State University School of Medicine, Columbus, Ohio.

*[Illustrations follow]*



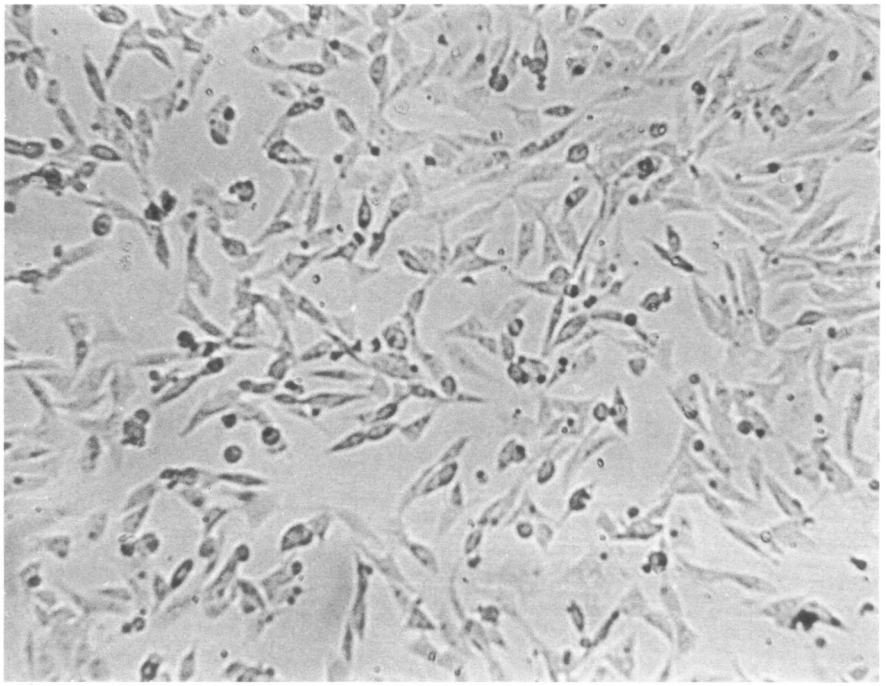
1A



1B

**Fig 1**—Macroscopic appearance of mouse L-cell fibroblast monolayers 48 hours after addition of test drop as described. **A**—L-cells cultured with 1 drop of guinea pig lymphoid cells (× 1). **B**—L-cells cultured with 1 drop of mixture of guinea pig lymphoid cells–concanavalin A (10 μg/ml). The area of cytotoxicity ("zone of clearing") is indicated by *arrows*.

2A



2B

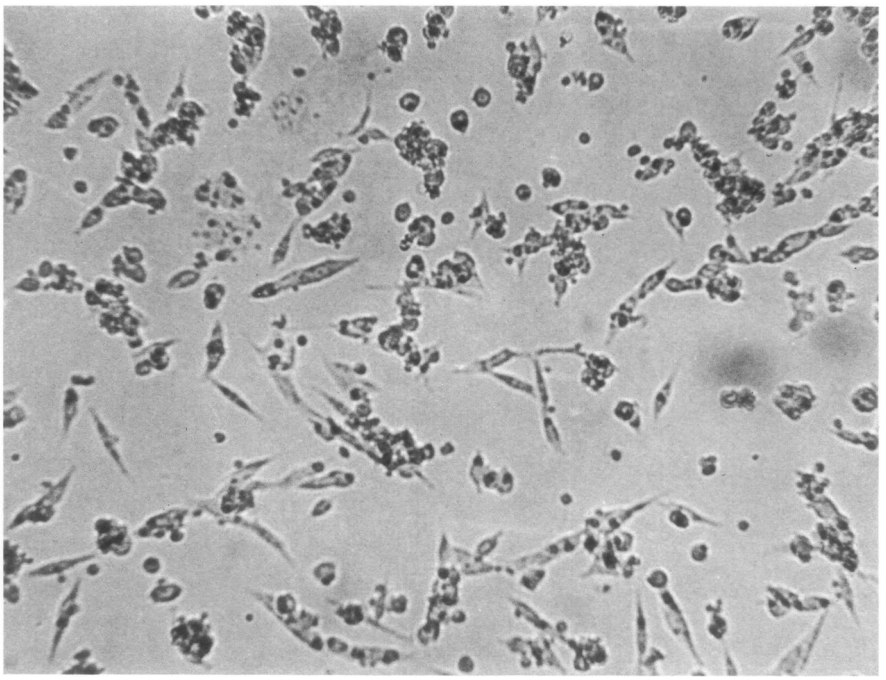


**Fig 2**—Appearance of mouse L-cell fibroblast cultures after 48-hour incubations as described. **A**—L-cells cultured in presence of guinea pig lymphoid cells. **B**—L-cells cultured in presence of mixture of guinea pig lymphoid cell–concanavalin A (10 μg/ml). Note the clustering of lymphoid cells about the target cells. Many target cells have been destroyed.