the oral dosage and was not significantly affected by germfree conditions. Growth rates indicated that a mineral level of 120% of the recommended daily allowances for conventional rats was adequate for germfree rats despite the fact that some of the minerals may be metabolized differently by rats in the two environments.

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## A Simple Micro Tissue Culture Method for the Determination of Lymphocyte Cytotoxicity *in Vitro** (33777)

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Different techniques have been used to assess the cytocidal effect on target cells *in vitro* of lymphocytes from nonimmunized and immunized donors. Monolayer cultures in different types of culture tubes have most commonly been employed (1–3), but methods utilizing the formation of plaques in petri dishes also have been described (4), as well as inhibition of colony formation (5). Common to most methods is the requirement of rather large numbers of lymphocytes. As supply of animals and cells used for immunization is often limited, a micro scale test is desirable. Our experience with a simple micromethod is described in the present report.

*Materials and Methods. Principle of the test system.* Mice were immunized with cultured cells. Cell suspensions consisting of known numbers of lymphocytes prepared from the lymphnodes of normal, *i.e.*, nonimmune, or sensitized animals were added to previously established monolayer cultures of known numbers of target cells. At specified time intervals after addition of lymphocyte suspensions, the number of target cells remaining in the cultures were quantitatively determined.

*Animals and procedure of immunization.* Young adult C3H/Fib mice of both sexes were employed for this study. The animals were sensitized by subcutaneous inoculation bilaterally in both flanks of a total of $20 \times 10^6$ cells usually suspended in 0.2 ml of Tyrode's solution. In some instances the cells were suspended in complete Freund's adjuvant. Hyperimmunization of the animals was achieved by 5–7 inoculations given at 7–10-day intervals.

*Target cells.* The three cultured cell lines used in the assays originated from normal C3H cells. The C3H-M cells were obtained from the spleen of an adult C3H mouse and the C3H-L cells came from the lung of the same mouse, whereas the C3H-E cells were obtained from cultures of 19-day-old C3H mouse embryos (6). All three cell lines had been propagated continuously *in vitro* for 4 years at the time of the present experiments.

The cells were propagated in Roux flasks on a standard medium consisting of 80% Eagle's minimum essential medium (7) with a twofold concentration of the essential amino acids and a fourfold concentration of the

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vitamins and glutamine to which was added 20% fetal bovine serum (FBS). The basic medium without FBS is designated Fib 41 B.

Cells used for immunization were harvested from the Roux flasks with a rubber policeman and resuspended at the desired concentrations in Tyrode's solution or Freund's complete adjuvant. Cells used as target cells in the assay were removed aseptically by means of 0.25% trypsin (Difco) in Hanks' balanced salt solution (HBSS). The cell suspension was gently centrifuged after trypsinization, the trypsin was discarded and the cells were resuspended in complete growth medium (Fib 41 B + 20% FBS). Counts were done in a Speirs-Levi eosinofil counting chamber after the addition of trypan blue dissolved in HBSS to give a final concentration of 0.05% trypan blue. Finally the cell suspension was diluted with complete growth medium according to the cell count to give a concentration of approximately 15 $\times$ $10^4$ live cells/ml of growth medium.

*Preparation of lymphocytes.* Immunized and nonimmunized animals were killed by dislocation of the cervical spine, usually 10 days after the last injection. Inguinal, axillary, and brachial lymph nodes were dissected free of the surrounding tissues under aseptical conditions and placed in a small amount of Fib 41 B in loose-fitting glass homogenizers (Braun-Melsungen) in which suspensions of lymphocytes were prepared by gentle grinding by hand. The suspensions were left for a few minutes to allow larger bits of tissue to sediment after which the lymphocyte and immunocyte suspensions were decanted off. The lymphocytes were washed three times in Fib 41 B and finally resuspended in the same fluid. After a trypan blue count the appropriate dilutions were made in Fib 41 B.

*Assay procedure.* For the micro tissue culture assay disposable plastic trays (Linbro Disposo Trays, FB-48 clear, obtained from the Flow Laboratories, Scotland) were employed. These trays were especially adapted for tissue culture. Each plastic tray contains 48 flat-bottomed cups each with a diameter of 6 mm and a volume of 0.5 ml. The trays were prepared by cutting off the edges and dividing each tray into four, each with 12 cups. The trays were washed in water with 7X detergent (Linbro Chemical Co.) and rinsed in running water. After washing they were immersed in 96% alcohol for 1 hr and finally rinsed in three changes of distilled water and air dried. The trays were sterilized by exposure to a UV lamp for 1 hr. After the sterilization they were aseptically placed in sterile, disposable plastic petri dishes with a diameter of 90 mm (Nunc, Petri dish, 90 mm, no. 1000-1). Target cells prepared as described above at a concentration of approximately 15 $\times$ $10^4$ cells/ml of complete growth medium, were seeded into each cup at a volume of 0.3 ml/cup. The cultures were incubated in a humidified incubator at 37° with a gas phase consisting of 5% $CO_2$, 20% $O_2$, and 75% $N_2$. After approximately 24 hr of incubation, the cells in 12 or 24 cups were enumerated. The tissue culture fluid was discarded and 0.2 ml of 0.25% trypsin was added to each culture. After 20 min of incubation at 37°, the cells were counted after vigorously pipetting the trypsin up and down several times in each culture to ensure a quantitative detachment of the cells from the bottom of the cups. The mean obtained from 12 or 24 cultures was used to calculate the desired number of lymphocytes to be added to the cultures. Ratios ranging from 20 to 100 lymphocytes to 1 target cell were usually employed. After discarding the culture fluid in the remaining cultures, lymphocyte suspensions adjusted to the proper concentrations in Fib 41 B without FBS were added to each culture, the control cultures receiving Fib 41 B alone. The volume added to each culture was 0.2 ml. Incubation was resumed in the humidified $CO_2$ incubator for 24 and 48 hr at which time cultures were counted in groups of 12 after trypsinization as described above.

*Results.* The results are summarized in Table I, which only gives the counts obtained after 48 hr of incubation with lymphocytes. In most instances counts obtained after 24 hr did not give significant differences between the cell numbers in cultures with nonimmune

TABLE I. The Influence of Sensitized and Nonsensitized Murine Lymphocytes on Three Target Cell Lines Grown in Disposable Plastic Trays.[a]

| Expt. no. and cell line | Treatment of donor mice | Ratio of lymphocytes to target cells | No. of living target cells × $10^3$ ($\bar{x} \pm$ SE) | CI[b] |
|---|---|---|---|---|
| 1. C3H-L | Immunized | 22:1 | 2.8 ± 2.4[e] | 0.79 |
| | Normal | 22:1 | 7.5 ± 4.6 | 0.44 |
| | | Control | 13.4 ± 7.2 | |
| 2. C3H-M[f] | Immunized | 30:1 | 3.0 ± 2.3[e] | 0.95 |
| | Normal | 30:1 | 8.4 ± 7.8 | 0.86 |
| | | Control | 61.3 ± 8.6 | |
| 3 C3H-M | Immunized | 25:1 | 5.4 ± 2.8[e] | 0.50 |
| | Immunized[c] | 25:1 | 3.8 ± 3.2[e] | 0.65 |
| | Immunized[d] | 25:1 | 4.5 ± 3.1[e] | 0.58 |
| | Normal | 25:1 | 17.9 ± 5.4 | 0.00 |
| | | Control | 10.8 ± 5.9 | |
| | Immunized | 50:1 | 3.6 ± 1.9[e] | 0.67 |
| | Immunized[c] | 50:1 | 3.8 ± 2.5[e] | 0.65 |
| | Immunized[d] | 50:1 | 3.2 ± 1.7[e] | 0.70 |
| | Normal | 50:1 | 10.8 ± 7.8 | 0.00 |
| | | Control | 10.8 ± 5.9 | |
| 4. C3H-M | Immunized | 25:1 | 18.7 ± 9.1 | 0.54 |
| | Immunized | 50:1 | 5.3 ± 3.7[e] | 0.87 |
| | Immunized | 100:1 | 1.3 ± 0.9[e] | 0.97 |
| | Normal | 25:1 | 20.3 ± 9.2 | 0.50 |
| | Normal | 50:1 | 13.9 ± 9.0 | 0.66 |
| | Normal | 100:1 | 2.8 ± 1.0 | 0.93 |
| | | Control | 40.3 ±11.7 | |
| C3H-L | Immunized | 25:1 | 13.8 ± 5.7[e] | 0.53 |
| | Immunized | 50:1 | 1.5 ± 0.7[e] | 0.95 |
| | Immunized | 100:1 | 0.1 ± 0.2[e] | 1.00 |
| | Normal | 25:1 | 35.3 ± 3.8 | 0.00 |
| | Normal | 50:1 | 13.8 ± 4.7 | 0.53 |
| | Normal | 100:1 | 3.7 ± 2.2 | 0.87 |
| | | Control | 29.3 ± 8.8 | |
| C3H-E | Immunized | 25:1 | 24.3 ± 9.8 | 0.48 |
| | Immunized | 50:1 | 3.5 ± 2.2[e] | 0.93 |
| | Immunized | 100:1 | 0 | 1.00 |
| | Normal | 25:1 | 19.5 ± 7.3 | 0.59 |
| | Normal | 50:1 | 13.4 ±11.6 | 0.72 |
| | Normal | 100:1 | 0.6 ± 0.5 | 0.99 |
| | | Control | 47.1 ±15.5 | |

[a] Results obtained in 4 different experiments using disposable trays after 48 hr of incubation with lymphocytes added to the monolayer of target cells. Student's $t$ test was used to test the significance at the 95% level when comparing the effect of immune lymphocytes versus normal, i.e., nonimmune, lymphocytes.

[b] CI (cytotoxic index) $= (C_c - C_e)/C_c$, in which $C_c$ is the mean number of target cells in the untreated control cultures and $C_e$ the mean number of target cells in the cultures treated with normal or sensitized lymphocytes.

[c] Cells used for immunization suspended in complete Freund's adjuvant before injection into the animals.

[d] Hyperimmunized.

[e] Significantly different from the comparable group with normal lymphocytes.

[f] FBS (5%) was added to the incubation medium in this experiment.

and immune lymphocytes except in case of high ratios of lymphocytes to target cells. Statistical significance was in all instances calculated by Student's $t$ test and determined at the 95% level. In testing for significance, comparisons were only made between cultures treated with normal lymphocytes and those treated with immunocytes with the same ratio of lymphocytes to target cells.

From Table I, it appears that lymphocytes from sensitized animals in all instances showed a significantly greater cytocidal effect on the target cells than the nonimmune lymphocytes. In some instances, however, the difference was only significant when ratios of 50:1 or 100:1 were used as in Expt. 4. Different methods used for sensitization of the donor animals did not influence the results obtained when adding immune lymphocytes to the cultures as is seen in Expt. 3. Addition of FBS to the incubation medium (Expt. 2) apparently did not influence the results. In some experiments, incubation of cultures was continued for 72 hr. In all instances it was found that both control cultures and cultures treated with lymphocytes and immunocytes had decreased so much in cell numbers that no meaningful counts could be made.

*Discussion.* Disposable micro plastic trays have been used in virology for the titration and neutralization of virus (8, 9). The use of these trays by Rosenbaum *et al.* resulted in tissue cultures suitable for microassays. However, it was found that reproducible assays could be obtained only when the toxicity of the plastic, resulting from molding, was eliminated by alcohol (95%) treatment of the plates. Our experience with the plastic trays also indicate that a meticulous cleaning is necessary and that the alcohol treatment is essential in order to obtain good results with the method. The present results showed that this method is satisfactory for the determination of lymphocyte cytotoxicity *in vitro*. The method is simple, reproducible and allows a quantitative estimation of the effect of the lymphocytes upon the target cells. It is easy with the plastic trays to operate with a large number of replicate microcultures, and the use of this method has substantially reduced the number of animals and cells required for tests of this type. With a concentration of 10–15 $\times$ $10^4$ target cells/cm$^2$, ratios of lymphocytes to target cells ranging from 20:1 to 50:1 are suitable and will give an answer in 24 or 48 hr. In several experiments, cultures with lymphocytes added have been kept for 72 hr. However, in almost all instances one finds a general decrease of cell number in cultures after 72 hr, usually of sufficient magnitude to rule out further useful results. It has frequently been observed, that even normal, nonimmunized lymphocytes show some cytotoxic effect. However, in all instances where cultures with normal and immune lymphocytes were compared, the latter always showed a significantly larger decrease in the number of target cells.

Preliminary results, not presented here, indicate that it is of some importance to determine the correct initial inoculum of the target cells. If too high an inoculum is used, the cultures will be overcrowded which may lead to an increased risk of cell death or detachment and loss of target cells when the cultures are manipulated during trypsinization before counting. The right initial inoculum will probably vary with the type of cells employed as target cells and will have to be determined in each instance. In the experiments here recounted, satisfactory results were obtained with an inoculum of approximately 3–5 $\times$ $10^4$ target cells/cup yielding a cell population of approximately 8–15 $\times$ $10^4$ cells/cm$^2$ after 24 hr of preincubation.

No differences were observed in the assays when incubation medium consisting of pure synthetic tissue culture medium Fib 41 B or medium with a small amount of FBS was used. The target cells usually remained healthy for 48 hr when incubated with synthetic medium without the addition of FBS. For enumeration of the target cells after the action of the lymphocytes, we have only used direct counting of the target cells after trypsinization of the cultures. It is believed that the trypsinization will result in digestion of dead cells and debris so the counts truly reflect surviving viable target cells (10).

From one of the experiments performed, it

appears that no difference is encountered in the action of the lymphocytes when different methods of immunization are used, *i.e.*, a single immunization with the cells suspended in Tyrode's solution, or in complete Freund's adjuvant or hyperimmunization. On the basis of the present observations we think that the micro tissue culture assay described here is a simple and useful tool for assaying the cytotoxic effect of lymphocytes *in vitro*.

*Summary.* A simple micro tissue culture method for the determination of the cytocidal effect of lymphocytes *in vitro* is presented. Suspensions of lymphocytes are added to monolayers of target cells in disposable plastic trays each containing 12 cultures with a volume of 0.2–0.3 ml, numbers of target cells per culture initially ranging from 3–5 $\times$ $10^4$ cells. Trypsinization of the cultures and enumeration of surviving target cells are done after 48 hr of incubation. The method is simple, requires small numbers of lymphocytes and target cells, and it is quantitative. It is concluded that this micro tissue culture method is well suited for the determination of the cytotoxic effect of lymphocytes *in vitro*.

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