Detection of human ADCC activity using automated flow cytometry

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Summary. Using automated flow cytometry techniques we have developed a rapid assay to measure human antibody-dependent cell-mediated cytotoxicity (ADCC). By staining cell cultures for DNA content, chick red blood cell targets can be readily distinguished from human effector cells and ADCC can be measured by changes in their relative proportions. The sensitivity and rapidity of the assay is shown by the finding that at an effector to target ratio of only 2:1, 42% killing can be detected after 1 h incubation.

Leukocytes of several types possess antibody-dependent cytotoxic potential (ADCC) and are called K cells[2]. K cell activity is thought to be an in vitro correlate of one of the tissue-damaging mechanisms arising in autoimmunity and in the course of an immune response to tumors, transplants or virally modified tissue[3].

K cell assays are most frequently performed by incubating effector cells from nonimmune donors with antibody coated-target cells. Many in vitro assays to measure K cell cytotoxicity have been described[3]. In addition, the K cell assay is highly useful for the functional characterization of lymphocytes in the blood or lymphoid organs of normal and diseased individuals.

The central need is for an assay that is technically reproducible, reasonably simple, quick to perform and applicable in a wide variety of experimental situations. In this report we have taken advantage of the difference in DNA content of target and effector cells to detect cytotoxicity using automated flow cytometry (FCM).

Materials and methods. Effector cells: Human peripheral blood lymphocytes (HPBL) were obtained from normal donors. Heparinized peripheral blood was layered on Ficoll-Hypaque and centrifuged for 40 min at 500 × g at 4 °C. Mononuclear cells removed and washed 3 times in RPMI 1640 media.

ADCC assay: 100 µl of HPBL were added to 2×10^5/50 µl freshly obtained chicken red blood cells (CRBC) in triplicate in Linbro 96-well roundbottom µl trays. In addition, either 50 µl of anti-CRBC (1:20,000 final dilution) or 50 µl media was added to the wells (total volume was 200 µl). This dilution of antibody was found to give optimal cytotoxicity. Plates were incubated at 37 °C in a 5% CO_2 incubator.

ADCC flow cytometry: Cultures were carried out as above with effector/target (E/T) ratios of 2:1, 1:1, and 1:2. The cultures were harvested at various times by aspirating the contents of one well into 1.0 ml of dye solution consisting of 50 µg/ml propidium iodide (PI), 0.1% trisodium citrate, 0.01 M NaCl and 0.1% Nonidet P-40. After equilibrating in the dye for at least 15 min at 4 °C the samples were analyzed by flow cytometry (FCM) on an Ortho Cytofluorograf model FC-200. In this instrument cells are individually illuminated by a 50 mW argon-ion laser (488 nm) at a rate of about 500 cells per sec; as each cell passes the laser beam it emits red fluorescent light proportional to its nuclear DNA content[4,5]. The signal generated by each cell was converted to a digital value (0–511) by an analogue-to-digital converter and stored in an Ortho 2102 multichannel analyzer. The resulting DNA histogram showed 2 distinct peaks corresponding to CRBC and effector cells. Each peak was integrated to obtain the number of cells in each peak, and the E/T ratio for each sample calculated from those values. The percent kill due to ADCC was calculated by the formula:

$$\% \text{ kill} = 100 - \frac{\text{E/T ratio control}}{\text{(E/T ratio of cultures with antibody to T)}} \times 100$$

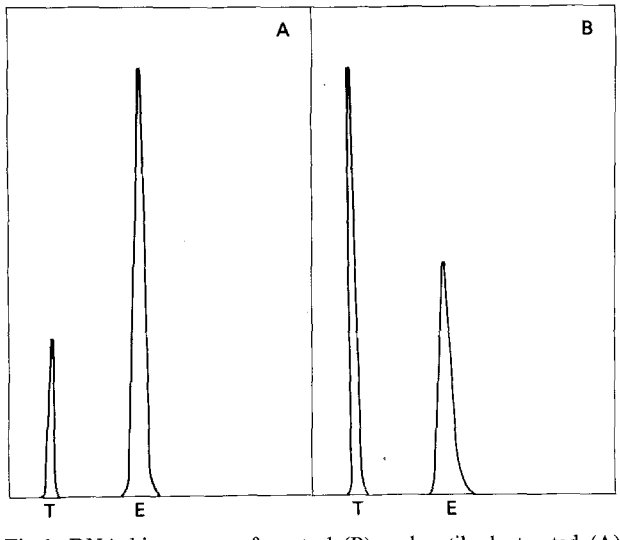

Fig. 1. DNA histograms of control (B) and antibody treated (A) cultures. The horizontal axis for each figure represents increasing DNA content while the vertical axis represents relative numbers of cells. Human effector cells have about 6.0 pg DNA/nucleus while CRBC have about 2.5 pg DNA/nucleus; thus, in each histogram effector cells (E) form the right hand peak while CRBC target cells (T) form the left hand peak.

Results and discussion. By using FCM one can rapidly determine the relative proportions of different cell types in a culture if they have distinctly different nuclear DNA contents such as CRBC and human mononuclear cells. Figure 1, A and B show representative DNA histograms from cultures with initial E/T ratio of 2/1 after 4 h

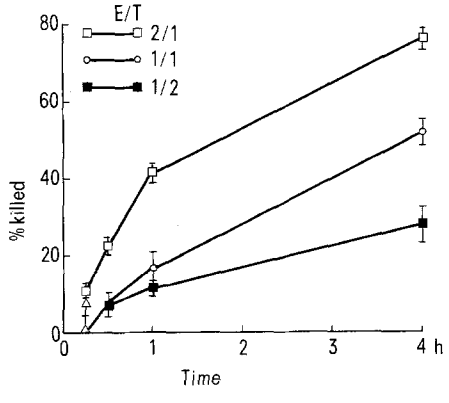

Fig. 2. Time course study of human ADCC at 2/1, 1/1 and 1/2 effector/target (E/T) ratios. Samples were harvested at 0.25, 0.5, 1 and 4 h. Each point shows the mean and SD of duplicate samples.

incubation. Figure 1, B represents the control culture without antibody. The peak to the left represents CRBC (T) while the peak to the right represents effector cells (E). The proportion of one cell type to another can be found by simply comparing the areas under the curve; in this case the E/T ratio is 12578/9833 or 1.2792. When antibody to CRBC is added to a culture some of the CRBC are lysed; as a result the proportion of CRBC to effector is decreased and the E/T ratio increases. Figure 1, A shows the change seen on a DNA histogram when anti-CRBC is added. Here the E/T ratio has increased to 21,010/3799 or 5.5304; the calculated percent kill is 77%.

Figure 2 shows the results of a time course study of ADCC by FCM using very low E/T ratios of 2/1 and less. The percent kill steadily increases over a 4-h span at all ratios resulting in a maximum kill of 77% at a 2:1 ratio and 52% at a 1:1 ratio. In addition, there is substantial killing (42%) after only 1 h at a 2/1 ratio.

When a standard chromium K cell assay was done using the same effector population we obtained 55% killing at a 50:1 E/T ratio after 4 h. Furthermore, plastic adherent cells showed enhanced cytotoxicity when measured by both FCM and chromium release[6], thus, FCM measures macrophage-monocyte killing.

This assay offers several advantages over the standard chromium release assay. First, no radiolabeling or gamma counting is needed. Second, substantial killing can be detected after only 1 h incubation compared to 4 h for the standard chromium assay. Third it is 50–100-fold more sensitive since much lower effector to target ratios than in conventional chromium assays can be used. Because of the extraordinary sensitivity of this system, this assay may be useful for the determination of antibodies against cell surface antigens, e.g., in connection with tissue typing or for detection of circulating immune complexes. Since K cell activity is mediated through the Fc receptor of the effector cell, antibody-coated cells or immune complexes can bind to effector cells and inhibit ADCC. For example, we have found that mouse and human serum containing immune complexes can significantly inhibit ADCC activity measured by FCM[7].

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Quantitative examinations of some rat proteins by means of absorbed anti-pregnancy and anti-feto sera

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Summary. This paper reports quantitative examinations of some rat serum proteins which occur only, or in rising concentrations, in pregnant rats before and after birth. A new serum protein is described which is only demonstrable in female rats. In male rats this protein is found rarely and then in very small quantities.

We examined quantitatively the development of some serum proteins of pregnant and lactating rats, as well as of young ones in their early phase of ontogenesis. This work was done in connection with research on mother-child interrelationships in the rat as a basis of quantitative and qualitative serum profile alterations.

Later on, we will report on quantitative variations of lipoproteins in the pregnant and lactating period of the rat. In this paper, we present new results concerning the quantitative behaviour of a pregnancy-associated protein (PAPP), a female specific protein (FP) and the alpha-feto protein (AFP) of the rat.

Pregnancy-associated proteins for the rat were described by Lin et al.[1,2] a few years ago. We have examined the time course of the protein which Lin et al. called PAPP-C. The position and shape of this protein peak permits a simple identification in crossed-immunoelectrophoresis (IE). Female-linked or -specific proteins are known at present for some insect species, for the hen and for the Syrian hamster[3]. Such a protein had not been demonstrated for the rat before.

Material and methods. The antisera directed against the PAPP's and against the FP were prepared according to Lin et al.[1]. Contrary to these authors, we absorbed the antisera separately with female and male serum, using normal serum, as well as lyophilized serum, for absorption purposes. Monospecific anti-AFP serum was prepared by means of absorption of an anti-feto serum with pooled serum of 8 weeks old female rats, into which turpentine was injected 48 h prior to exsanguination according to Ganrot[4] to give a high serum alpha-2-macroglobulin level. This is important for the preparation of a monospecific anti-AFP serum, because feto serum has a high amount of alpha-2-macroglobulin.

We applied the following analytical methods: rocket-IE[5], crossed-IE[6], fused-rocket-IE[7] and tandem-IE[8]. Serum of pregnant rats (21st day of pregnancy; normal rat gestation lasts about 22–23 days) was gel-filtered using Sephadex G-200. The animals, adult female and male Wistar rats of different ages, were obtained from Falcke-Barby. The fetal and young rats were obtained from the rat breeding department of the institute. The sera of the animals, pooled and single, were prepared in the usual way[1]. The immunization of the rabbits was accomplished according to the scheme of Harboe and Ingild[9].

Results and discussion. The quantitative examinations of the sera of fetal and young rats revealed a high level of AFP in 20-day-old fetuses. The protein concentration decreased with the increasing age of the young rats and was no longer demonstrable 8 weeks after birth by means of