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A RAPID MICROFILTER TECHNIQUE FOR DETERMINATION OF TRITIATED THYMIDINE INCORPORATION INTO DNA OF PERIPHERAL LYMPHOCYTES.

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INTRODUCTION.

In the past few years tests for in vitro proliferative response to antigenic, mitogenic (such as phytohemagglutinin) and allogeneic cells stimulation are extensively employed in clinical immunology and has since became one of the most important criteria for establishing the status of cellular immunity of individual. Among several available methods available, the scintillation counting for determination of the radioactive thymidine incorporation into the DNA of lymphocytes is the most widely used assay techniques (1-14). These assay techniques require centrifugation of radioactive cells, acid precipitable material and/or chemical digestion of DNA. These manipulations are not only time-consuming but also introduce variables which adversely affect the reproducibility and applicability of the assay itself. In addition, the amount of blood sample required for these conventional techniques was too great particularly for the study in pediatric patients. Thus the simple and reliable microtechnique becomes necessary.

Recently Robbins et al (15) described the technique in which a millipore filter is used to trap a sample's radioactive cells, essentially as was employed for the measurement of small amount of ^{3}H-TdR incorporation into the DNA of UV-irradiated lymphocytes (16). We share the experience with Robbins et al (17) that with slight modification this millipore technique is a simple and sensitive assay of ^{3}H-TdR incorporation into the DNA of lymphocytes in the leukocyte cultures stimulated with PHA.

We are describing a rapid microfiler technique of the millipore filter method which is simple, reprodu-

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cible, and requires only a very small amount of blood or leukocyte suspension which is currently used in one laboratory.

METHOD.

BLOOD SAMPLES. Fresh heparinized venous blood (20 units heparin per ml. blood), leukocyte-rich plasma (LRP) and lymphocyte suspension are prepared as previously described (14, 18). Aliquots of leukocyte suspension or blood sample (25–200 microliters) are transferred into sterile 10 × 75 mm. plastic-capped culture tubes (Falcon Plastics, Oxnard, Calif.) containing 25 ul. of PHA-P. (Difco Laboratories, Detroit, Mich.) and appropriate amount of Hank's-Hepes-Buffered medium (supplemented with 20% decomplemented fetal bovine serum, 100 units of penicillin/ml, and 50 mg. of streptomycin/ml.) to the final cuhtre volume of 1.5 ml.. The culture tubes are tightly stoppered and incubated at 37°C for 72 to 96 hours. All samples are carried out in triplicates.

The degree of proliferative response to PHA stimulation of lymphocytes was quantitated by determination of ^{3}H-TdR uptake. At the end of incubation period, 0.05 uCi ^{3}H-TdR (Amersham, Buckinghamshire, England) was added to each culture tubes. Unless otherwise stated, after 18 hours at 37°C, the whole content of the culture tube is transferred into the receiving funnel of the filtering apparatus.

FILTERING APPARATUS. (FIGURE 1) A Whatman glass fiber filter paper GF/A (2.4 cm., circular, W and R. Ballton LTD., England) is placed in the Pyrex Microanalysis Filter Holders with fritted glass base. The whole set is tightly held together with a spring clamp and serve as the receiving funnel.

The funnel is fitted through a rubber stopper into standard 250 ml. vacuum flask. A short length of rubber tubing is fitted to the lower most end of the funnel within the flask to prevent suctioned fluid from escaping through the flask's sidearm. Tubing from the flask is connected to a vacuum source.

HARVESTINN AND WASHING OF CELLS. After loosening the cells from the culture tube's wall by vortex, the content or its aliquot is transferred into the filtering funnel and apply vacuum to start filtration. The culture tube is flooded with 5 saline washes before they are applied to the filter to remove the remaining culture fluid and

adherent cells in the tube. The filter and the cells trapped on it are then washed with two successive 1 ml. aliquots of 0.9% saline and 10 ml. of 0.5 M.Perchloric acid. The damp filter is then placed flatly at the bottom of a clean counting vial.

PREPARING FILTER FOR SCINTILLATION COUNTING. The counting vial containing a damp filter is placed in a drying oven in vacuo at 80 °C for 15 minutes. Thereupon, the dried filter is wetted by adding to the counting vial 1 ml of a scintillation fluid (14). Radioactivity on the filter is then determined in a Model 4320 Packard Tri-Carb Scintillation Spectrometer (Packard Instrument Co., Downers Grove, Ill.).

OBSERVATIONS.

Difficulting in filtration is frequently observed in culture containing whole blood volume over 50 ul.. In this situation a known aliquot of culture sample is filtered then calculated back for the total culture activity. When the leukocyte suspension is used the optimum total incubation time is 120 to 144 hours but 96 hours is adequate. The peak ^{3}H-TdR incorporation is observed with the ^{3}H-TdR exposure time of 18 hours then declined significantly at 24 hours. When the same aliquot volume of whole blood and leukocyte suspension are used it is observed that the degree of ^{3}H-TdR incorporation of the latter is about 20 times more than the former. This may partly due to the higher number of lymphocytes in the latter condition but mainly may be due to the adsorption of PHA on to the red cell membrane when whole blood is used resulting in the lower amount of PHA available for lymphocyte stimulation.

COMMENTS.

The filter paper disc procedure for radioassay of in vitro DNA synthesis was originally developed by Bollum(19) and later extended to in vitro assay of protein synthesis(20). This technique was furthermodified by Byfield and Scherbaum(21) to assay the nucleic acid and protein synthesis of whole cells. Evans and Norman(16) were the first to introduce technique using millipore filter collecting radioactive cells for the scintillation spectroscopic measurement of UV-induced ^{3}H-TdR incorporation in human peripheral blood lymphocyte which later was used in the determination of ^{3}H-TdR incorporation into the DNA of mouse L cells during semiconservative DNA

synthesis [22]. More recently Robbins et al [15,17] reported a successful modified technique in which a Millipore filter with its retained radioactive cells, after being suitably washed and dried, is immersed in scintillation fluid and precisely positioned with respect to ensure a highly efficient and reproducible counting geometry for every filter.

The modified millipore and glass filter paper technique is rapid, since the use of whole blood avoids time-consuming purification of lymphocytes. Apart from the advantage of time saved, the use of nonseparated blood cells in natural proportion should better reflect the immunologic reactivity of the subject. Further more, this micromethod can be performed with as little as 0.1 ml. volume of blood, thus allowing one to perform a large number of tests with limited amounts of blood such as in young children or newborn infants. When very low level of radioactivity is used, counting efficiency can be increased at least 2 fold by combusting the filters and their radioactivity contents to tritiated. water [17]. The sensitivity of the assay techique can be measured by comparing the radioactivity obtained from well stimulated cultures to the unstimulated cultures. Our results indicate that the radioactivity uptake of the former is 50-100 times more than those of the latter (or the proliferation index of 50-100) comparable to results obtaind from the conventional technique [23]. This technique is highly reproducible since the standard deviation of the results obtained are proportional to the mean with the variation coefficient varies from 3% to 15%, mostly below 10% which is also comparable to those observed by others [24,25]. More than 99% of the radioactivity trapped on the filter was removed after treatment with DNase indicated that it is a very sensitive and specific assay or DNA synthesis in the leukocyte culture [15,37]. It has been observed by us and others [17] that the radioactivity uptake of the culture were linearly proportional to the amount of leukocytes and volune of culture fluid applied to the filter only if they are of small quantity. When large quantity of sample were used the radioactivity uptake obtained were well below the expected value extrapolated from those of smaller volume sample. These observations indicated that well counting efficiency is constant for smaller aliquots of culture, it decreases as the number of cells trapped on the filter is increased, most likely

due to increasing degrees of beta ray absorption by the excessive layers of cells deposited on the filters[17].

SUMMARY.

A simple, rapid and sensitive microfilter assay technique measuring tritiated thymidine incorporation during DNA synthesis of peripheral lymphocytes culture is described. The test is applicable to both the whole blood and leukocyte suspension. Lymphocytes which have been incubated with ^{3}H-TdR are trapped on a glass fiber or millipore filter which is then suitably washed, dried and its radioactive content counted. This technique is suitable in aiding the clinical evaluation of immune deficiency states where only small amount of blood sample is available.

ย่อเรื่อง

คณะผู้รายงาน ได้ทำการศึกษาและทดลองเกี่ยวกับการวัดปริมาณ Tritiated thymidine incorporation ระหว่างที่มีการสังเคราะห์ DNA ของ Peripheral lymphocytes culture โดยใช้วิธี Microfilter assay ซึ่งให้ผลรวดเร็ว, sensitive และเป็นวิธีที่ทำได้ง่าย การทดสอบใช้ได้ทั้ง wholeblood และ leukocyte suspension

เมื่อนำเอา leukocytes มา incubate ด้วย ^{3}H-TdR มันจะจับเอา ^{3}H-TdR ในระหว่างที่อยู่บน glass fiber หรือ millipore filter จากนั้นจึงนำไปล้าง ทิ้งไว้ให้แห้งแล้วนำไปนับปริมาณ radioactivity วิธีการตรวจชนิดนี้ เหมาะสำหรับใช้เป็นการประเมินผลทางคลินิคของภาวะพร่องภูมิคุ้มกันของร่างกาย ซึ่งต้องการปริมาณเลือด ในการตรวจจำนวนเล็กน้อยเท่านั้น.

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