Proc. Nat. Acad. Sci. USA
Vol. 72, No. 2, pp. 474–478, February 1975

Demonstration That Monocytes Rather Than Lymphocytes Are the Insulin-Binding Cells in Preparations of Human Peripheral Blood Mononuclear Leukocytes: Implications for Studies of Insulin-Resistant States in Man

(phagocytosis/adherence columns/autoradiography/receptors)

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Communicated by Irving M. London, November 7, 1974

ABSTRACT **Insulin receptors have been demonstrated on mononuclear leukocytes prepared by centrifugation of buffy coats from normal blood donors on Ficoll–Hypaque gradients. The cell type that specifically binds insulin in this mixture of lymphocytes and monocytes has never been clearly identified, although it was assumed to be the lymphocyte since this cell constitutes about 80% of the population. In the present studies, insulin-binding assays were performed on the mononuclear leukocyte preparation before and after selective depletion or enrichment for monocytes using glass wool or Sephadex G-10 adherence columns. The amount of ^{125}I-labeled insulin specifically bound correlated significantly with the number of monocytes but not with the number of B or T lymphocytes. Approximately 90% of the specific insulin binding of this preparation could be accounted for by its content of monocytes. The amount of binding was unaffected by phagocytosis of latex particles or by metabolic inhibitors added to prevent endocytosis. Autoradiograms made on smears of whole peripheral blood and mononuclear leukocytes demonstrated that all of the cells that bound ^{125}I-labeled insulin were large mononuclear cells, 85–90% of which could be identified as monocytes by morphological criteria or by the functional criterion of latex particle ingestion. Since insulin receptor concentration may be altered in disease states in man, it is essential, when using this cell population for detecting such changes, to quantitate the number of monocytes in the preparation so that the insulin-binding data can be appropriately interpreted.**

Previous studies by Gavin, Archer, and their coworkers (1–4) demonstrated that bone marrow-derived (B) lymphoblastoid cell lines maintained in continuous culture and peripheral blood mononuclear leukocytes from normal blood donors purified by Ficoll–Hypaque gradient centrifugation contain receptors for insulin. In the latter case, it was assumed that the binding was to receptors on lymphocytes, which comprise the majority of this population. However, a subsequent study by Krug *et al.* (5) failed to detect any significant insulin binding to peripheral blood lymphocytes purified by passage over tightly packed nylon wool. Since this separation procedure has recently been shown to remove B lymphocytes (6–9), it was suggested by Olefsky and Reaven (10) that the insulin binding measured in preparations of mononuclear leukocytes might have been to receptors on B lymphocytes. On the other hand, monocytes can constitute a significant portion of peripheral blood mononuclear leukocytes (11), and these cells are also depleted in preparations made using nylon wool (5, 12). Thus, the identity of the mononuclear leukocyte that binds insulin remained an open question.

In the present work we address ourselves directly to this problem. By various depletion and enrichment experiments we show that specific insulin binding correlates with the number of phagocytic cells (monocytes) in peripheral blood mononuclear leukocytes and not with the number of B lymphocytes or other cell types. In addition, direct visualization of the cells binding ^{125}I-labeled insulin by autoradiography demonstrated that these cells are predominantly monocytes both by morphological and functional criteria.

MATERIALS AND METHODS

Cell Preparations. Peripheral blood (300–500 ml) from normal human volunteers was drawn into acid–citrate–dextrose solution and centrifuged at 1500 × *g* for 3 min at 20°. The buffy coat was removed, diluted 1:2 with phosphate-buffered saline (pH 7.2), and fractionated on Ficoll (Pharmacia, Piscataway, N.J.)–Hypaque (Winthrop, New York, N.Y.) gradients (ρ = 1.077) according to the method of Böyum (13). The resulting interfaces were pooled and the cells (henceforth referred to as mononuclear leukocytes) were washed and counted. Viability, as assessed by trypan blue dye exclusion, was always greater than 95%. The B lymphoblastoid cell line, IM9, which has been shown to have a large number of insulin receptors, was kindly supplied by Dr. Donald Buell (14–16).

Identification of Monocytes and Lymphocytes. Monocytes were identified by morphological criteria (17) in cytocentrifuge smears stained with Giemsa's stain and by the functional criterion of latex particle ingestion (18). B lymphocytes were identified by the use of fluorescein-conjugated, rabbit anti-human immunoglobulin (Cappel Laboratories, Downingtown, Pa., lot no. 6684) (19). Cells were stained after exposure to latex particles and only nonphagocytic, fluorescent cells were considered positive. Thymus-derived (T) lymphocytes were identified by sheep erythrocyte rosette formation (20).

Depletion and Enrichment of Monocytes. Monocytes were depleted from mononuclear leukocytes by two adherence techniques. The first involved passage of the cells over col-

Abbreviations: B and T lymphocytes, bone marrow-derived and thymus-derived lymphocytes, respectively.

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umns of glass wool (21). Two grams of washed (0.2 M HCl and distilled water) Pyrex wool were packed tightly into a 20-ml plastic syringe and further washed with Eagle's minimal essential medium containing 10% fetal calf serum. Cells (5×10^8 in 5 ml of medium) were loaded onto the column at room temperature. The cells were washed through the column without incubation, using 50 ml of medium, and the nonadherent, monocyte-depleted population was collected (yield, 20–30%). The second technique involved passage of the mononuclear leukocytes over columns of Sephadex G-10. The procedure described by Ly and Mishell (22) was used except that the bed of glass beads was replaced with a small amount (<40 mg) of glass wool. The yield of cells in the nonadherent, monocyte-depleted population was 35–60%.

A monocyte-enriched population was also obtained from the Sephadex G-10 columns. This was accomplished after removal of the nonadherent cells by dispersing the Sephadex in excess medium and mechanically agitating them with a Vortex mixer to remove the adherent cells (yield, 20–30% of the cells put onto the column).

Insulin-Binding Assay. Porcine insulin (Eli Lilly, Indianapolis, Ind.) was iodinated to a specific activity of 100–200 μCi/μg (2). Binding studies were performed as described (2, 3) with the exception that 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 8, replaced 25 mM Tris, pH 7.4. The cells were incubated with insulin for 3 hr at 22° and then separated from the unbound insulin. The specific ^{125}I-labeled insulin in the cell pellets was determined as described in Table 1.

Autoradiography. Cells were incubated for 3 hr at 15° in the presence of 6–20 ng of ^{125}I-labeled insulin, washed three times through cold fetal calf serum adjusted to pH 8, smeared onto gelatin-coated slides, and fixed with 1% (v/v) glutaraldehyde in phosphate-buffered saline. Autoradiograms were then prepared by the method of Davie and Paul (23). Cells were considered to have insulin receptors if four or more silver grains above background were observed over or closely surrounding the cell. In several experiments the cells were allowed to ingest latex particles prior to the binding of ^{125}I-labeled insulin.

RESULTS

In 10 experiments the average composition of the mononuclear leukocytes prepared from peripheral blood buffy coats by Ficoll–Hypaque gradient centrifugation was 22.2 ± 2.6% (range 10–36) monocytes, 8.6 ± 1.7% (range 2–16) B lymphocytes, and 68 ± 2.6% (range 58–79) nonphagocytic, non-immunoglobulin-bearing cells. The last category was shown to consist almost exclusively of T lymphocytes by the criterion of rosette formation with sheep erythrocytes. The mononuclear leukocytes also contained a few contaminating granulocytes (about 1%) and a variable number of platelets. Erythrocyte contamination never exceeded one erythrocyte per 10 leukocytes. The average specific insulin binding of this mixed population was 5.1 ± 0.7% (range, 3.8–7.9) of the 0.1–0.5 ng of ^{125}I-labeled insulin added to the cells.

To ascertain the role of monocytes in this insulin binding, mononuclear leukocytes were selectively depleted of these cells by adherence techniques. When the percentage of monocytes was reduced from 27.5% to 1.3% by passage of the mixed population over glass wool columns, the specific insulin binding was reduced from 5.9% to 1.2% (Table 1). In contrast, the percentage of immunoglobulin-bearing cells (B lymphocytes) remained unchanged and the percentage of nonimmunoglobulin-bearing, nonphagocytic cells increased. Thus, depletion of 95% of the monocytes resulted in an 80% decrease in the specific insulin binding. If the remaining pha-

TABLE 1. *Binding of ^{125}I-labeled insulin to mononuclear leukocytes separated on glass wool and Sephadex G-10 adherence columns*

Cell populations*	% Specific insulin binding†	% Monocytes‡	% B lymphocytes§	% Nonimmunoglobulin-bearing, nonphagocytic cells
Glass wool columns				
Unseparated cells	5.9 ± 2¶	27.5 ± 8.5	4 ± 2	68.5 ± 10.5
	(3.8–7.9)	(19–36)	(2–6)	(58–79)
Nonadherent cells	1.2 ± 0.3	1.3 ± 0.3	4 ± 3	94.7 ± 3.3
	(0.3–1.5)	(1–1.7)	(1–7)	(91–98)
Sephadex G-10 columns				
Unseparated cells	4.7 ± 0.5	23.2 ± 4.9	8.3 ± 3	65.5 ± 3.5
	(4.0–6.1)	(16–32.5)	(3–15)	(60–72)
Nonadherent cells	0.79 ± 0.03	2.0 ± 1.5	6.3 ± 1.3	90.7 ± 2.3
	(0.72–0.88)	(0.33–5)	(3–9)	(86–93)
Adherent cells	7.9 ± 1.4	39.0 ± 3	8.7 ± 2	50.5 ± 1.5
	(5.4–11.7)	(36–42)	(1–12)	(49–52)

* Unseparated cells are the mononuclear leukocytes derived from Ficoll–Hypaque gradient separation of human peripheral blood buffy coats. The nonadherent cells were those mononuclear leukocytes that were not retained by the glass wool or Sephadex G-10 matrix after the columns were washed with 50 ml of medium. The adherent cells from Sephadex G-10 were those cells that were retained after washing but removed by subsequent mechanical agitation.

† Cells (4 to 8 × 10^7) were incubated with 0.1–0.5 ng of ^{125}I-labeled insulin for 3 hr at 22°, pH 8, in the presence or absence of 50,000 ng/ml of unlabeled insulin. The percentage of ^{125}I-labeled insulin specifically bound was calculated by subtracting the percentage of ^{125}I-labeled insulin bound in the presence of unlabeled insulin (nonspecific) from the percentage bound in the absence of unlabeled insulin (total).

‡ Phagocytic cells were quantitated by the criterion of latex particle ingestion. These cells were almost entirely monocytes, as most of the granulocytes had been removed during the Ficoll–Hypaque separation.

§ B lymphocytes were quantitated by staining with fluorescein-conjugated rabbit anti-human immunoglobulin. Only those stained cells that had not ingested latex particles were considered positive.

¶ All values are expressed as mean percentages ± SEM, with the range in parentheses.

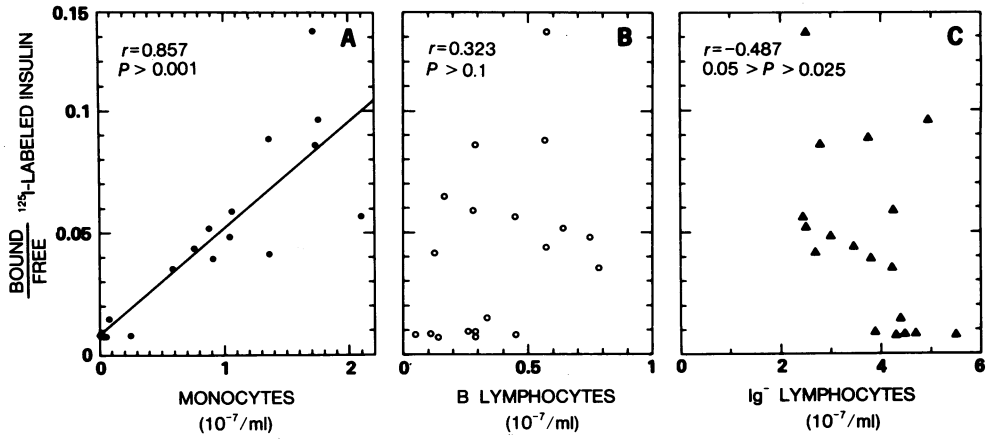

FIG. 1. Correlation between the quantitative level of insulin binding and the number of monocytes. Monocytes were quantitated by latex particle ingestion (A) and B lymphocytes by staining with fluorescein-conjugated rabbit anti-human immunoglobulin (B). The remaining cells were classified as nonimmunoglobulin-bearing, nonphagocytic cells (Ig$^-$ lymphocytes in C). Insulin binding was carried out as described in the footnotes to Table 1. Correlation coefficients (r) were calculated under the assumption that the paired data points were a random sample from a bivariate normal distribution. In the case of the monocytes (A), the best-fitting straight line was drawn through the data points using a linear regression analysis of Y (insulin binding) on X (cell number).

gocytic cells had been removed, the binding would have been expected to decrease another 4%.

Mononuclear leukocytes were also depleted of monocytes by passage over Sephadex G-10 columns (Table 1). When the percentage of monocytes in the mixed population was reduced from 23.2% to 2.0%, the specific insulin binding was reduced from 4.7% to 0.79%. Again the percentage of B lymphocytes remained essentially unchanged and the percentage of nonimmunoglobulin-bearing, nonphagocytic cells was increased. Based on these figures, the monocytes account for 91% of the binding.

A major advantage of the Sephadex G-10 separation procedure is that it allows the recovery of a substantial portion of the cells that adhere to the column; this population is usually enriched for phagocytic cells. As can be seen in Table 1, the specific insulin binding and the number of monocytes in the adherent population showed parallel increases relative to the unseparated population of cells. In contrast, the percentage of B lymphocytes was the same for the two populations and the percentage of nonimmunoglobulin-bearing, nonphagocytic cells declined. The demonstration that the adherent cells recovered from the column have functional insulin receptors suggests that the lack of binding in the nonadherent population was not the result of blockage or disruption of the insulin receptors by the columns. Further support for this point was obtained by passage of the lymphoblastoid cell line (IM9) through a Sephadex G-10 column. The cells that emerged in the nonadherent population showed as much specific insulin binding (15.1%) as the unseparated cells (12.5%).

Fig. 1 is a compilation of all the experiments in which both insulin binding and the number of monocytes or B lymphocytes were determined. These include unseparated cell populations as well as the separated populations from both glass wool and Sephadex G-10 experiments. The quantitative level of insulin binding correlated significantly ($P < 0.001$) with the number of monocytes (Fig. 1A). The slope (0.89) of the best-fitting straight line was not significantly different from the slope (1.0) of the line obtained on serial dilution of a population containing a fixed number of monocytes. There was no correlation with the number of B lymphocytes (Fig. 1B) and an inverse correlation with the number of nonimmunoglobulin-bearing, nonphagocytic cells (Fig. 1C). The latter merely reflects the absence of monocytes in this population as a result of the derived nature of the data (by subtraction). Thus, of the two major cell types in mononuclear leukocytes, monocytes rather than lymphocytes account for most of the insulin binding.

Although the rapid kinetics of insulin dissociation from monocytes (A.R.B., manuscript in preparation) made it unlikely that the monocytes were simply ingesting insulin–receptor complexes shed from other cells, this possibility was ruled out by studying insulin binding in the presence of metabolic inhibitors to block endocytosis (24, 25). Both 3 mM sodium azide and 0.1 mM iodoacetamide either alone or in combination failed to prevent binding of ^{125}I-labeled insulin. Cell viability in both treated and control groups was greater than 90% at the end of the incubation. Conversely, phagocytosis of latex particles by monocytes did not affect insulin binding. Both the degree of specific binding and the inhibition of binding of ^{125}I-labeled insulin by various amounts of unlabeled insulin were identical for mononuclear leukocytes preincubated either with or without latex particles.

Visualization of the cells that bind ^{125}I-labeled insulin by autoradiography directly demonstrated insulin receptors on monocytes (Fig. 2) in both mononuclear leukocytes and whole peripheral blood. All of the cells seen with silver grains over them could be morphologically classified as large mononuclear cells. Most of these cells (>85%) could be positively identified as monocytes; however, the remaining cells with silver grains could not be distinguished from large lymphocytes. In addition, there appeared to be a small number of large mononuclear cells that did not have silver grains associated with them. Small lymphocytes, neutrophils, and eosinphils did not show any binding by this technique. The study with whole blood ruled out the possibility that the procedure used for preparing mononuclear leukocytes had resulted in a selective loss of subpopulations of lymphocytes or granulocytes that might otherwise have bound insulin under these conditions. Similar autoradiograms were observed when the binding of insulin was carried out in the presence of metabolic inhibitors. Slides made of cell preparations in which the binding of ^{125}I-labeled insulin was carried out in the presence of a large excess

of unlabeled insulin showed no localization of grains around any cell type, at the exposure times examined (1–3 weeks), indicating that the binding was specific.

To further characterize the cell type that bound insulin, the mononuclear leukocytes were allowed to phagocytize latex particles prior to insulin binding and autoradiography. As noted above, the ingestion of latex particles did not decrease the specific insulin binding, indicating that the insulin receptor was apparently not modulated by phagocytosis. Silver grains were localized over and around those cells that had ingested the latex particles (Fig. 2). Ninety percent of the cells binding insulin were latex-positive. The remaining 10% could be morphologically characterized as large mononuclear cells. Only 14% of the latex-ingesting cells showed no insulin binding. Again, competition experiments with unlabeled insulin eliminated the localization of silver grains. Thus, the predominant cell type in mononuclear leukocytes that binds insulin appears to be a phagocytic cell with the morphologic characteristics of a monocyte. In addition, there appears to be a small population of nonphagocytic, large mononuclear cells that also bind insulin.

DISCUSSION

The purpose of this study was to identify the insulin-binding cell present in Ficoll–Hypaque preparations of mononuclear leukocytes from human peripheral blood. Fractionation of this population by several techniques showed a highly significant positive correlation between insulin binding and the number of monocytes, but no positive correlation with the number of lymphocytes, red blood cells, or platelets. Depletion of monocytes from the mixed population by use of glass wool or Sephadex G-10 adherence columns reduced the insulin binding by 80–90% with no consistent change in B or T lymphocytes. Recovery of the cells adherent to the Sephadex G-10 column gave a population of cells that was enriched for monocytes and that showed an increased amount of insulin binding.

Since granulocytes are similar to monocytes with respect to phagocytic and adherence properties, it was possible that the small number of contaminating granulocytes (1%) in mononuclear leukocytes accounted for the majority of the insulin binding. This possibility was ruled out by a direct examination of the cells binding ^{125}I-labeled insulin using autoradiography. Silver grains were found in significant numbers only over large mononuclear cells, greater than 85% of which could be identified as monocytes by morphology and the functional criterion of latex particle ingestion. Silver grains were never seen in significant numbers over small lymphocytes or granulocytes. Thus, the cell responsible for the major portion of the insulin binding in mononuclear leukocytes appeared to be the monocyte.

Receptors on monocytes could only account for 90% of the specific insulin binding by mononuclear leukocytes. The remaining 10% of the binding was seen by autoradiography to be to nonphagocytic, large mononuclear cells. It is possible that this subpopulation of insulin-binding cells represents nonphagocytic, nonadherent monocytes. On the other hand, these cells might be circulating large lymphocytes. Such cells have been shown in the rat (26) to be mainly dividing precursors of intestinal plasma cells, i.e., blast-transformed or activated B lymphocytes. In this regard, Krug *et al.* (5) reported that lectin-activated lymphocytes acquire receptors for insulin.

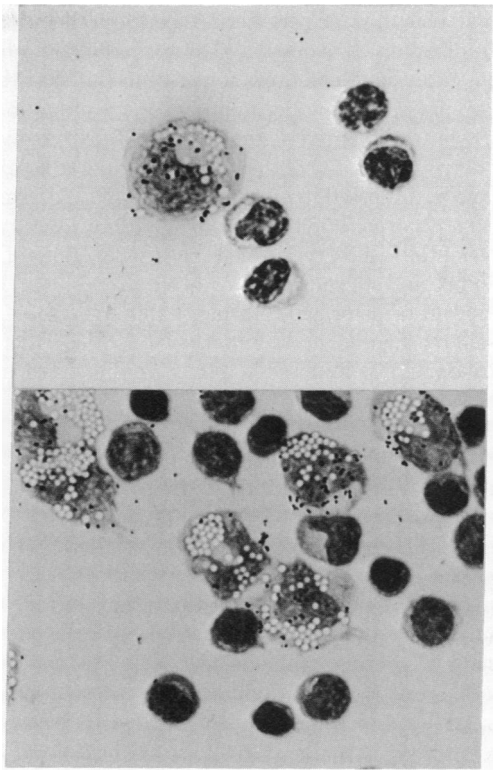

FIG. 2. Localization of ^{125}I-labeled insulin binding to mononuclear phagocytes by autoradiography. Mononuclear leukocytes were first incubated with latex particles for 30 min at 37°. The cells were then exposed to 12 ng/ml of ^{125}I-labeled insulin for 3 hr at 15°, pH 8, after which they were washed several times through fetal calf serum, pH 8, at 4° and smeared onto slides. Autoradiograms were prepared and developed after 3 weeks of exposure. The latex particles appear in the photographs as small white beads in the cytoplasm of the monocytes. The ^{125}I-induced silver grains appear as black dots. Magnification is 800×. The figure at the top demonstrates a monocyte with more than 20 grains and four lymphocytes with no grains; the lower figure illustrates the same distribution of ^{125}I-labeled insulin in a larger field of mononuclear cells.

About 15% of the monocytes appeared not to bind insulin by autoradiography, suggesting the existence of a subpopulation of monocytes. One possibility is that the two monocyte populations represent cells in different parts of the cell cycle, since differential expression of surface antigens has been reported in various stages of the cycle (27). On the other hand, the lack of binding could simply be due to a technical artifact, such as thinning of the emulsion over some cells or loss of ^{125}I-labeled insulin from receptors during the washing procedure.

The major lesson to be learned from these studies is that in any mixed population one should not assume that the predominant cell type is necessarily responsible for hormone binding or any other biochemical function under study. When possible, it is important to quantitatively correlate the binding with changes in cell composition or with other specific markers of cell function and to localize the binding to the particular cell type by autoradiography or other techniques. With regard to these points, the presence of insulin receptors has been reported on granulocytic leukemia cells and cultured lymphoblastoid cells as well as on normal granulocytes and thymocytes (1–4, 28). In contrast, our autoradiograms failed to de-

tect similar receptors in peripheral blood granulocytes and B and T lymphocytes. It is possible that normal blood cells other than monocytes have receptors for insulin, but that these receptors are present in smaller numbers or are of lower affinity than those on the monocyte, or require different assay conditions for detection. In any of these cases, binding to the receptor might have been missed in our autoradiograms. Alternatively, it is possible that contaminating monocytes or histiocytes are the source of the insulin receptors in the preparations of normal granulocytes and thymocytes reported by others. Finally, neoplastic and transformed cells may exhibit surface receptors not present on their normal counterparts. Until further experiments are done, it is impossible to resolve the discrepancies in these various studies.

Finally, we should consider how these findings affect the usefulness of the human mononuclear leukocyte preparation in the study of insulin receptors in man. Alterations in the insulin–receptor interaction have been observed in liver (29) and adipose tissue (30) of animals with diseases in which there is increased or decreased insulin sensitivity. The insulin receptor on the monocyte is indistinguishable from those in liver and fat by multiple criteria (refs. 31 and 32; A.R.B., manuscript in preparation) and provides a readily accessible source of material for studying hormone–receptor interactions in man. Archer *et al.* (3, 4) and Olefsky and Reaven (33) have demonstrated decreased insulin binding to mononuclear leukocytes from patients with some insulin-resistant states and diabetes. In light of the present results, it becomes essential when studying such patients to quantitate the number of monocytes in the preparation. In several patients with marked insulin resistance, previously reported to have a decrease in insulin receptors (4), our more recent studies demonstrate that normalization of insulin-binding data to monocyte number actually increases the apparent magnitude of the insulin receptor deficiency. Recognition that the monocyte is the major insulin-binding cell in the mononuclear leukocyte preparation should allow one to better define the insulin receptor deficit previously reported in obesity (3) and to detect even more subtle alterations in the insulin–receptor interaction in other disease states by eliminating a previously unsuspected variable.

We thank Mrs. W. S. Chappell, Mrs. F. J. Shoup, and Mrs. V. B. Weber of the NIH Blood Bank for drawing the blood and preparing the buffy coats used in these experiments. In addition, we thank Mr. John Williams for technical assistance and Drs. Jesse Roth and William Terry for many stimulating discussions and advice. A.R.B. is a recipient of PHS Fogarty International Fellowship FO5 TW 1964-01.

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