INCORPORATION OF ^{75}Se-SELENOMETHIONINE IN HUMAN PERIPHERAL LYMPHOCYTES AS AN IN VITRO TEST OF GLOBULIN-SYNTHESIZING CAPACITY

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At one time, circulating lymphocytes were regarded as mature cells at the terminal stage. In 1960 Nowell (*1*) discovered mitosis of normal human leukocytes in response to phytohemagglutinin, an extract of kidney bean (*Phaseolus vulgaris*). Since then stimulation of lymphocytes has been observed as increases in synthesis rates of DNA (*2*), RNA (*3*) and proteins (*4*) in the presence of this mitogenic agent. These studies were based on the incorporation of various isotopically labeled compounds such as thymidine, uridine and amino acids. The isotopes used in these cases have commonly been beta emitters. Forbes and Henderson (*5*) used ^{14}C-amino acids to study globulin synthesis of lymphocytes in culture. They found that lymphocytes obtained from patients with diseases such as chronic lymphatic leukemia, Waldenstrom's macroglobulinemia and some other immunologic disorders do not respond to phytohemagglutinin as much as lymphocytes obtained from normal human subjects (*5*). We have adopted lymphocyte culturing as a diagnostic assay using a labeled compound with a gamma emitter. We find that the amino acid analog, ^{75}Se-selenomethionine, is satisfactory for this investigation.

This paper describes an assay in which the globulin-synthesizing capacity of human peripheral lymphocytes has been measured in terms of the incorporation of ^{75}Se-selenomethionine into the globulin protein. Results of lymphocyte cultures on twenty normal human individuals are presented.

METHODS

Lymphocyte suspensions. Ten-milliliter blood samples were drawn from normal adults in heparinized syringes and then transferred into sterilized capped culture tubes containing 0.3 ml of heparin (heparin sodium, 1,000 U.S.P. units/cc). Five-tenths milliliter of 6% w/v Dextran in saline was added. The tubes were inverted five times to insure mixing and the mixture was allowed to settle for 1.5 hr at 37°C. The leukocyte-containing plasma layer above the red-cell boundary were withdrawn and transferred to a sterile prescription bottle. With the flat side of the bottle lying on a leveled shelf, the cell suspension was incubated at 37°C for 1 hr. Granulocytes became attached to the glass surface. The plasma layer containing the lymphocytes was withdrawn and centrifuged at 900 g for 15 min. The supernatant or platelet-rich plasma was withdrawn and used to prepare the plasma in the way described in the next section. The lymphocyte-rich pellet was washed with Tissue Culture Medium 199 and resuspended in a culture medium for plating.

Culture medium. Tissue Culture Medium 199 supplemented with 20% of autologous human plasma was used. The autologous plasma was separated from platelet-rich plasma by sedimentation at 1,250 g for 15 min. ^{75}Se-selenomethionine (4 μCi in 1 ml; obtained from the Radiochemical Centre, Amersham) was added to 12 ml of the culture medium.

Phytohemagglutinin (PHA) from Burroughs Wellcome was dissolved in 5.0 ml of sterile distilled water. One milliliter of this stock solution was arbitrarily designed as 1.0 unit of activity. The stock solution was stored in a frozen state.

Assay procedure. Replicate cultures of lymphocytes were prepared by suspending about 3×10^6 cells in 1.5 ml of culture medium in a sterile disposable capped culture tube. The culture medium contained 0.3 μCi of ^{75}Se-selenomethionine in 1.5 ml. To certain designated tubes 0.05 ml of PHA was added. Duplicate cultures with and without PHA were incubated for 24 hr at 37°C. In addition, there were duplicate cultures for zero-time controls. All cultures were terminated by adding sodium sulfate to make a concentration of 18%

Received April 19, 1968; original accepted June 14, 1968.
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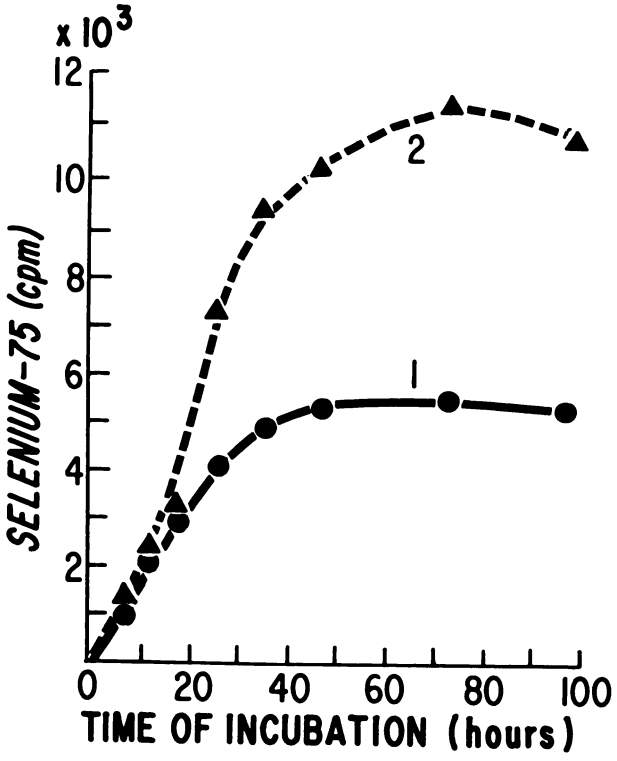

FIG. 1. Effect of varying periods of incubation with phytohemagglutinin on incorporation of ^{75}Se-selenomethionine into globulin protein of lymphocytes in culture. In Curve 1 replicate cultures were incubated in PHA-free medium. In Curve 2 replicate cultures were incubated in medium containing 0.05 units of PHA. Capacity of such cultures to incorporate ^{75}Se-selenomethionine into globulin protein was assayed at end of incubation period. Response in terms of counts per minute is plotted against the total hours of exposure of each culture.

for the precipitation of globulin protein. The precipitate was washed three times with 16% sodium sulfate. The radioactivity of the globulin was measured in a gamma well scintillation spectrometer. Radioactivity of 24-hr cultures without PHA (corrected for zero-time controls) represented the amount of ^{75}Se-selenomethionine incorporated into unstimulated cells. Radioactivities of 24-hr cultures with PHA represented the stimulated cell metabolism. The ratio of radioactivities of stimulated to unstimulated cells represents an index of lymphocyte stimulation by PHA.

RESULTS

To establish parameters of the assay procedure, we assessed duration of culturing, PHA concentrations and the temperature effect.

Using the dye exclusion technique (trypan blue) we found that lymphocytes undergoing the separation procedure remained viable. Culturing for 24 hr at 37°C without PHA reduced the viability of lymphocytes to about 85%. Under the same conditions, PHA seemed to affect the viability in variable degrees. The viability of PHA-treated lymphocytes ranged from 40 to 80%.

Figure 1, which depicts a time-course curve, shows that stimulation of lymphocytes by PHA began at about 16 hr and then progressed rapidly. The curve reached a plateau and then declined, perhaps because the PHA or tissue culture medium was exhausted. This was later confirmed by experiments in which further addition of PHA resumed the stimulation measured by the incorporation of ^{75}Se-selenomethionine.

Figure 2 shows the temperature effect. Of the three temperatures studied—4°, 23°, and 37°C—37°C resulted in the greatest stimulation.

When normal human peripheral lymphocytes were cultured in the 24-hr assay system with varying levels of PHA, the dose-response curve shown in Fig. 3 resulted. No significant stimulation of globulin synthesis occurred when 0.003 units of PHA were added. Above this level the response increased rapidly until it reached a plateau, indicating a saturation level of PHA. In a number of experiments, high levels of PHA resulted in a falling off of the response curve. In our case, a response plateau occurred only after 0.03 or more units of PHA were added.

To assess the reproducibility of the assay procedure, we tested replicate cultures of peripheral lymphocytes collected from a clinically normal subject. Cultures with and without PHA were incubated at 37°C for 24 hr. Radioactivity of ^{75}Se-

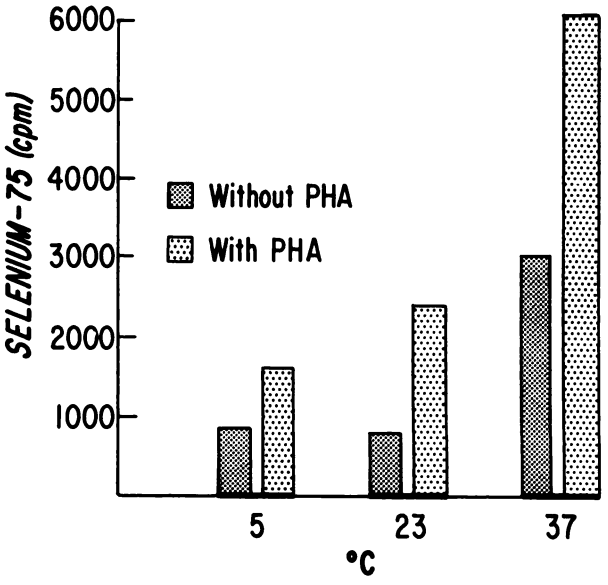

FIG. 2. Temperature effect on the incorporation of ^{75}Se-selenomethionine into globulin protein of lymphocytes in culture with and without phytohemagglutinin. Lymphocytes were cultured for 24 hr in medium containing 0.5 μCi of ^{75}Se-selenomethionine. Tubes with PHA contained 0.05 units of phytohemagglutinin.

selenomethionine incorporated into the globulin protein of 10^6 lymphocytes is given in Table 1. Standard deviations were calculated. We found that, in unstimulated cultures, an amount of ^{75}Se-selenomethionine emitting 3,090 ± 128 cpm was incorporated into globulin protein per million lymphocytes incubated for 24 hr. Cultures stimulated with PHA incorporated an amount giving 9,060 ± 265 cpm. Thus, the calculated index of stimulation by PHA was 2.94 ± 0.18. These results indicate satisfactory reproducibility of the assay procedure.

Tests were made on cultures of lymphocytes collected from a normal human subject on different days. Results from six samples are shown in Table 2. Unstimulated cultures had counting rates of 3,779 ± 628 cpm of ^{75}Se-selenomethionine incorporated into globulin protein of 10^6 lymphocytes incubated for 24 hr, while cultures stimulated with PHA gave 8,706 ± 2,160 cpm. The calculated index of stimulation with PHA was 2.32 ± 0.50.

To establish ranges of the globulin-synthesizing capacity of peripheral lymphocytes in a normal population, samples obtained from 20 clinically normal individuals were tested. As Table 3 shows, in unstimulated cultures an amount of ^{75}Se-selenomethionine emitting 2,971 ± 878 cpm was incorporated into globulin protein of 10^6 lymphocytes incubated for 24 hr, while cultures stimulated with PHA gave 7,780 ± 1,819 cpm. The index of stimulation by PHA was 2.75 ± 0.63.

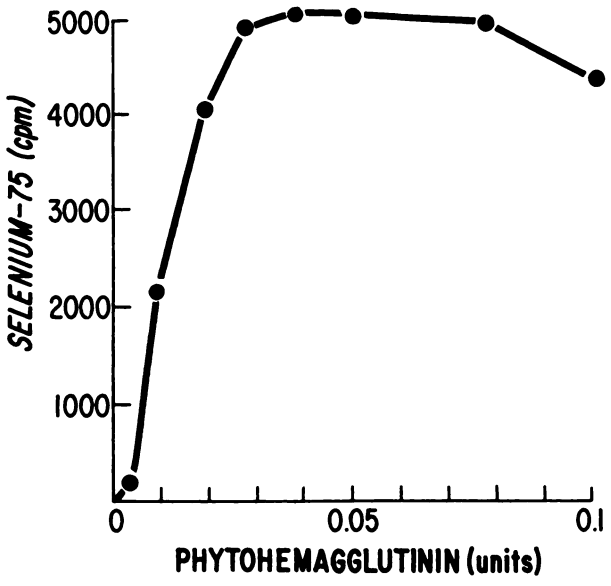

FIG. 3. Effect of varying concentrations of phytohemagglutinin on incorporation of ^{75}Se-selenomethionine into globulin protein of lymphocytes in culture at 37°C for 24 hr.

TABLE 1. REPRODUCIBILITY OF VALUES FOR INCORPORATION OF ^{75}Se-SELENOMETHIONINE INTO GLOBULIN PROTEIN OF LYMPHOCYTES IN CULTURE AT 37°C FOR 24 HR (LYMPHOCYTES FROM A NORMAL INDIVIDUAL)

Tube	^{75}Se counting rate (cpm)/10^6 lymphocytes		Index of Stimulation†
	Unstimulated*	Stimulated*	
1	3,280	9,003	2.74
2	3,006	8,927	2.97
3	3,129	8,852	2.83
4	2,946	9,456	3.21
Mean value	3,090	9,060	2.94
± s.d.	128	265	0.18

* Cultures stimulated with phytohemagglutinin.
† Index of stimulation = stimulated cultures/unstimulated cultures.

TABLE 2. REPRODUCIBILITY OF VALUES FOR INCORPORATION OF ^{75}Se-SELENOMETHIONINE INTO GLOBULIN PROTEIN OF LYMPHOCYTES IN CULTURE AT 37°C FOR 24 HR (LYMPHOCYTES OBTAINED FROM A NORMAL INDIVIDUAL ON DIFFERENT DAYS)

Date	^{75}Se counting rate (cpm)/10^6 lymphocytes		Index of stimulation
	Unstimulated*	Stimulated*	
1-18-67	3,459	6,713	1.94
1-30-67	2,782	5,506	1.98
4-5-67	4,329	8,774	2.04
4-24-67	4,724	8,969	1.90
6-12-67	3,859	12,108	3.14
7-13-67	3,519	10,167	2.89
Mean value	3,779	8,706	2.32
± s.d.	628	2,160	0.50

* Average of duplicate cultures.

DISCUSSION

Peripheral lymphocytes are a population of cells with restricted metabolic activities. In culture these cells can be stimulated by mitogenic agents such as phytohemagglutinin (*1*) as well as by certain antigens (*6*). Functional abnormalities may be related to certain diseases such as lymphoproliferative disorders, hematopoietic disturbances and immunologically deficient syndromes (*7–10*). Forbes and Henderson (*5*) have demonstrated a decrease in lymphocyte stimulation by PHA in cases of leukemia and other diseases. They studied the incorporation of ^{14}C-amino acids into sodium sulfate precipitated globulin protein in 24-hr lymphocyte cultures. We adopted the same principle in studying the globulin-synthesizing capacity of lymphocytes, except that we used ^{75}Se-selenomethionine. With the aim of developing lymphocyte-culturing into a feasible diagnostic procedure, we have tested the

reproducibility of results, the accuracy and the sensitivity of this method. We made repetitive analyses on replicate aliquots of a lymphocyte suspension from a human subject as well as on samples obtained on different days from a human subject and on twenty clinically normal individuals. With this assay procedure it has been possible to establish a normal range of stimulation index (ratio of stimulated culture to unstimulated culture). Variations of observed results may be experimental and/or biological, and deviation may be reduced with refinement of the method. The coefficient of variation* for day-to-day changes in the same individual is almost as large as the differences between individuals in a normal population. Since the coefficient of variation for duplicate analyses is less than ⅓ of these other coefficients, it suggests a considerable biologic variability within an individual which should be of physiologic interest.

In developing this assay method, we have also studied several parameters including PHA concentrations, lymphocyte densities and the time course of cultures. Results have enabled us to choose optimal conditions for the assay.

When selenomethionine was mixed with cells, we have found some nonspecific interaction between the labeled compound and cells. Similarly, Blau (*11*) has observed selenomethionine-protein interaction. In our case, reduced temperature (at 4°C) did not decrease such a reaction.

When lymphocytes were cultured with selenomethionine in a medium that did not contain phytohemagglutinin, there was an uptake of the labeled compound, indicating a basal metabolism of lymphocytes. When these cells were stimulated by the mitogenic agent PHA, the incorporation of selenomethionine into globulin protein was increased. ^{75}Se-labeled globulin protein (separated with sodium sulfate) was allowed to react with rabbit antisera against human serum proteins. A major precipitin arc on immunoelectrophoretic patterns had an electrophoretic mobility similar to that of gamma globulin. Furthermore, radioautography revealed that this major precipitin arc carried the radioactivity.

It has been reported that the type of serum used in the lymphocyte culture plays an important role in the response to PHA (*12*). The variations observed in our series of 20 clinically normal individuals may be due in part to inhibitory principles which might be present in some sera.

Forbes and Henderson (*5*) used autologous plasma in their assay. There is a possibility of an interfering effect on the PHA-stimulating system of some inhibiting principles which might be present in pathologic sera. Therefore it is preferable to carry out cultures of lymphocytes from patients both in autologous plasma and in clinically normal donors' plasma, heparinized and processed in a similar fashion.

It is known that lymphocytes in the presence of PHA do not synthesize DNA during the first 24 hr. This is the period during which there is rapid acceleration of RNA and protein synthesis. We have observed that in lymphocyte cultures there was a latent period of 16 hr for the PHA-stimulating effect to become manifest. It appears to indicate the need of having PHA in contact with the cell to set in motion the events leading to protein synthesis.

The assay method we describe requires only 10 ml of blood and a period of 24 hr for lymphocyte culturing. Radioactivity measurement for gamma-emitting ^{75}Se is convenient. Therefore the technique seems simple enough to become a routine laboratory procedure for *in vitro* studies of globulin synthesis.

TABLE 3. RANGE OF NORMAL VALUES FOR INCORPORATION OF ^{75}Se-SELENOMETHIONINE INTO GLOBULIN PROTEIN OF LYMPHOCYTES IN CULTURE AT 37°C FOR 24 HR (LYMPHOCYTES FROM 20 NORMAL INDIVIDUALS)

Subject	^{75}Se counting rate (cpm)/10^6 lymphocytes		Index of stimulation
	Unstimulated*	Stimulated*	
1	2,470	8,141	3.34
2	3,442	9,216	2.68
3	2,940	5,341	1.82
4	2,568	7,855	3.06
5	4,892	9,452	1.93
6	4,187	9,715	2.32
7	1,865	5,256	2.82
8	4,344	9,001	2.27
9	2,537	9,856	3.89
10	2,427	9,979	4.11
11	2,641	7,473	2.83
12	1,331	4,504	3.38
13	2,531	6,503	2.58
14	3,648	7,970	2.18
15	1,900	5,788	3.05
16	3,395	9,458	2.79
17	3,674	9,571	2.61
18	3,016	6,698	2.22
19	2,645	4,756	1.80
20	2,752	9,070	3.30
Mean value	2,971	7,780	2.75
± s.d.	878	1,819	0.63

* Average of duplicate cultures.

* Coefficient of variation = (Standard deviation/mean) × 100. Coefficient of variation for individual day-to-day reproducibility is 0.50/2.32 = 0.215. Coefficient of variation between individuals in a normal population is 0.63/2.75 = 0.229. Coefficient of variation for duplicate analysis is 0.18/2.94 = 0.06.

However, one must be careful to have consistent experimental conditions and to use reliable reagents. Aseptic conditions should also be followed strictly. Although it is acceptable, the reproducibility of the method must be improved further.

SUMMARY

An *in vitro* assay procedure has been described for measuring the globulin-synthesizing capacity of human peripheral lymphocytes in the presence of ^{75}Se-selenomethionine and phytohemagglutinin (PHA). Lymphocytes cultured at 37°C for 24 hr incorporated small amounts of ^{75}Se-selenomethionine into globulin protein in unstimulated cultures, while lymphocytes in the presence of PHA incorporated a much greater amount of this amino acid analog in stimulated cultures. The ratio between radioactivities of stimulated cultures and unstimulated cultures gave an index of stimulation by PHA on the globulin-synthesizing capacity of lymphocytes. Values of such an index of stimulation by PHA for lymphocyte cultures obtained from twenty clinically normal individuals were 2.75 ± 0.63. Several parameters and reproducibility of the assay method were also tested.

ACKNOWLEDGMENTS

This investigation was supported by a grant from the Iowa Division, American Cancer Society and in part by a grant from the James Picker Foundation on recommendation of the Committee on Radiology, National Academy of Sciences, National Research Council. We thank F. Benoit for his interest in this work and for his help in getting blood samples from normal human subjects. The technical assistance of Mrs. Margaret Horn and Miss Martha Ollivier is also acknowledged.

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