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The Formation of Immunoglobulins by Human Tissues *in vitro*

II. QUANTITATIVE STUDIES

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Summary. The present paper deals with some of the quantitative aspects of immunoglobulin formation *in vitro*. The results of this study show that isolated spleen cells synthesize about 1·25 μg IgG per 10^8 lymphoid cells during the first 6 hours of incubation. The initial rate of synthesis was not maintained throughout the 48 hours of incubation, but decreased markedly after 6 hours.

Comparison of the intensity of the autoradiographic line with the amount of radioactive immunoglobulin in the precipitation line revealed that a very distinct line (gradation +++) corresponds to about 0·0125 μg IgG. The limit of visibility (+) is reached at approximately 0·0002 μg radioactive IgG.

INTRODUCTION

Assessment of the synthesis of immunoglobulins by means of immunoelectrophoresis in combination with autoradiography (Hochwald, Thorbecke and Asofsky, 1961; van Furth, Schuit and Hijmans, 1966) provides semi-quantitative information, when carried out under standard conditions. An attempt was therefore made to obtain more information concerning the amount of immunoglobulin synthesized *in vitro* and the quantity of protein detected by autoradiography.

The present investigations were carried out with lymphocyte suspensions from monkey spleens (*Macaca cynomolgus*) because insufficient human tissue was available for duplicate experiments. The antigenic similarity between the immunoglobulins of primates and man (Picard, Heremans and Vandebroek, 1962) made it possible to use antisera against human immunoglobulins.

METHODS AND MATERIALS

Spleen cells

Spleens of two healthy monkeys, killed with chloroform, were used. The tissues were minced in Hanks' solution, and a suspension of lymphoid cells was made with a Borel type of tissue press (Fagraeus, 1963 personal communication; Balfour, Cooper and Alpen, 1965).

Amino acid pool

A known number of lymphoid cells was washed with buffered saline and extracted with 5 per cent trichloroacetic acid after gentle homogenization in a Potter–Elvehjem homogenizer. The cellular proteins and particles were removed by centrifugation for 10 minutes

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at 1500 ***g***, after which the sediment was again extracted twice with 5 per cent trichloroacetic acid. The trichloroacetic acid in the combined supernatants was extracted with ether, and the ether then removed by evaporation. The amino acid solution was concentrated by lyophilization. The total amount of amino acids was determined colorimetrically according to Folin (1922). The lysin content was determined with a Technicon amino acid Auto-Analyzer.

Culture experiments

Five cultures, each containing a known number of lymphoid cells and 1 ml culture medium, were incubated for 6, 12, 24 or 48 hours, at 37°. The culture medium for these experiments was the same as described in the previous paper (van Furth *et al.*, 1966), but contained only 1 μc/ml [^{14}C]lysine (specific activity 124 μc/m-mole; Schwarz Bio Research, Orangeburg). Cold isoleucine was added to a final concentration of 0·3 m-mole/ml. At the end of the incubation period the cultures were frozen (−20°) and thawed, centrifuged for 20 minutes at 18,000 ***g***, and the supernatant dialysed against a 0·015 M/phosphate buffer (pH 7·6) for 96 hours at 4°.

In four culture fluids the synthesized IgG was precipitated and prepared for counting as described by Thorbecke (1960). To correct for non-specific adsorption of radioactive materials on the IgG precipitate, the culture fluids were first absorbed twice with a precipitate of diphtheria toxoid and horse anti-toxin at equivalence. This precipitate contained about 1·75 mg protein. The first precipitate was discarded; the second was counted. The IgG precipitate was then prepared by adding to the culture fluid a small amount of purified human IgG (0·2 ml of a 0·8 per cent solution) and a specific rabbit anti-human IgG antiserum in slight excess (0·2 ml of an antiserum in a concentration of 0·75 mg antibody N/ml). The immune precipitate contained at equivalence approximately 1·0 mg protein. The precipitates were washed three times with phosphate-buffered saline (pH 7·2), treated with 5 per cent trichloroacetic acid at 90° for 15 minutes to remove interfering radioactive components, and then dissolved in 1 ml of 98 per cent formic acid. The samples were transferred to aluminium planchettes, dried, weighed and then counted in an automatic-recording, gas-flow counter with a windowless Geiger tube (Tracerlab., Inc.). Corrections for resolving time were applied according to Taylor (1951), and the corrections for self-adsorption were calculated from an empirical curve. On the assumption that the IgG precipitates carry down about the same amount of radioactivity non-specifically as the diphtheria toxoid precipitates, the counts of the IgG precipitate were corrected by subtracting the counts of the second diphtheria toxoid precipitate.

The culture medium was counted in duplicate under the same conditions. For this purpose 0·1 ml culture medium was added to about 1·0 mg IgG immune precipitate. From these data the percentage of [^{14}C]lysine incorporated into IgG was determined and the amount of synthesized IgG was calculated.

The fifth culture, which contained the same number of cells, was incubated for 48 hours, dialysed as described previously, concentrated by lyophilization, and dissolved in 0·1 ml twice distilled water. This culture was used for comparative quantitative autoradiographic studies.

Additional experiments were carried out with spleen-cell suspensions obtained from two other monkeys. Two samples of these suspensions, containing $0{\cdot}3\times10^8$ and $0{\cdot}6\times10^8$ lymphoid cells, were incubated for 1½, 3 and 6 hours in 1 ml of a medium containing two radioactive amino acids, as used routinely. Two other samples, both containing

$0{\cdot}6 \times 10^8$ lymphoid cells, were pre-incubated for 3 or for 6 hours at 37°, with 1 ml of a medium in which the two radioactive amino acids were replaced by an equal amount of unlabelled amino acids. After pre-incubation this medium was replaced by 1 ml radioactive culture medium and the cultures were incubated again for 6 hours. After incubation all the cultures were treated and counted as described above.

Immunoelectrophoresis and autoradiography

The concentrated culture fluids of samples used for comparative autoradiographic studies were diluted with phosphate-buffered saline (pH 7·2) in serial two-fold dilutions. Immunoelectrophoresis was carried out as described in the previous paper (van Furth *et al.*, 1966), with minor modifications. Both antigen wells (1 μl) of each slide were first filled once with normal human serum and then three times with the same dilution of the culture fluid. The immune precipitates were developed with a specific antiserum against human IgG. The slides were washed for 5 days in buffered saline, after which the two identical immune precipitates on the slide were separated. One-half of the agar was transferred to another microscope slide, dried, stained and exposed exactly 3 weeks for the autoradiography.

The precipitation line in the other half of the agar was cut out and transferred to a test tube containing 0·5 ml distilled water. The protein was then extracted from the agar as described by von der Decken (1963). After the agar gel had been dissolved by heating for 3 minutes at 90°, the protein was extracted and precipitated with 0·5 ml 10 per cent trichloroacetic acid at 90°. The precipitated proteins were centrifuged for 10 minutes at 1500 ***g*** twice re-treated with 5 per cent trichloroacetic acid at 90°. The protein precipitate was then dissolved in 0·5 ml 98 per cent formic acid, transferred to aluminium planchettes, and counted as described in the preceding paragraph.

From these data the percentage [^{14}C]lysine in the IgG isolated from the precipitation line was determined, and the total amount of [^{14}C]IgG calculated. This result was compared with the gradation of the autoradiographic pattern of the identical immune precipitate on the same slide.

RESULTS

The total amount of amino acids in spleen samples 1 and 2 was 46·7 and 41·7 μg per 10^8 lymphoid cells; the lysine content of these samples amounted 2·54 and 3·29 μg per 10^8 lymphoid cells. One millilitre of medium contains 1·18 μg [^{14}C]lysine, so the total amount

TABLE 1

AMOUNT OF IgG SYNTHESIZED BY LYMPHOID CELLS OF THE SPLEEN

Incubation time (hours)	Spleen No. 1*		Spleen No. 2†	
	[^{14}C]Lysine incorporated (%)	Equivalent amount of synthesized IgG (μg)	[^{14}C]Lysine incorporated (%)	Equivalent amount of synthesized IgG (μg)
6	2·30	1·07	1·73	0·85
12	2·52	1·17	2·13	1·05
24	3·02	1·41	1·50	0·74
48	3·09	1·44	1·74	0·86

* Per culture $0{\cdot}83 \times 10^8$ lymphoid cells.
† Per culture $0{\cdot}70 \times 10^8$ lymphoid cells.

of lysine per culture is known. The cultures of spleen 1 ($0{\cdot}83 \times 10^8$ lymphoid cells) contain 3·29 μg lysine and those of spleen 2 ($0{\cdot}7 \times 10^8$ lymphoid cells) contain 3·48 μg lysine. Human IgG contains 7·06 per cent lysine (Crumpton and Wilkinson, 1963) and it was assumed that this figure did not differ significantly for monkey IgG, so that the amount of

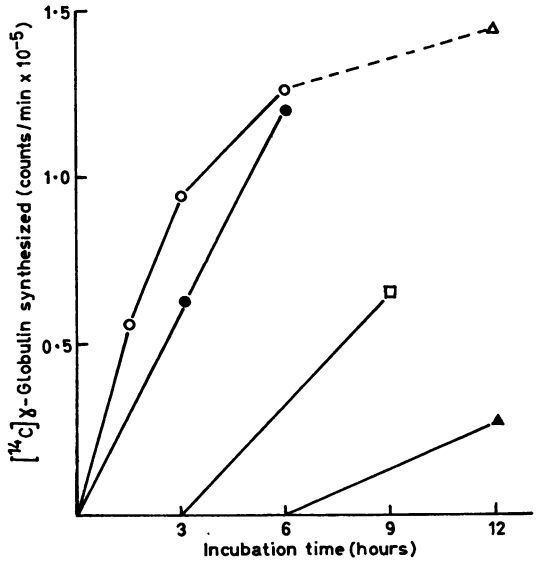

FIG. 1. Synthesis of γ-globulin during the initial hours of incubation. ○, $0{\cdot}6 \times 10^8$ lymphoid spleen cells, incubated with 1 ml radioactive medium; ●, $0{\cdot}3 \times 10^8$ lymphoid spleen cells, incubated with 1 ml radioactive medium; □, $0{\cdot}6 \times 10^8$ lymphoid spleen cells, pre-incubated for the first 3 hours with a non-radioactive medium subsequently replaced by 1 ml radioactive medium; ▲, $0{\cdot}6 \times 10^8$ lymphoid spleen cells, pre-incubated for the first 6 hours with a non-radioactive medium subsequently replaced by 1 ml radioactive medium; ○ - - - △, calculated from data presented in Table 1.

IgG synthesized *in vitro* could be calculated (Table 1). These results show that the major amount of IgG is synthesized during the first 6 hours of incubation. This synthesis continued to increase slightly in spleen 1 up to 24 hours and in spleen 2 up to 12 hours.

TABLE 2

COMPARISON OF THE AMOUNT OF IgG PRECIPITATED ON THE IMMUNOELECTROPHORESIS SLIDE WITH THE INTENSITY OF THE AUTORADIOGRAPHIC IMAGE FROM THE CULTURE FLUID OF LYMPHOID SPLEEN CELLS

Dilution of culture fluids*	Spleen No. 1		Spleen No. 2	
	IgG precipitated† (μg)	Autoradiography‡	IgG precipitated† (μg)	Autoradiography‡
1 : 1	0·0120	+++	0·0135	+++
1 : 4	0·0033	++	0·0021	++
1 : 8	0·0017	++	0·0013	+
1 : 16	0·0008	+	0·0010	+
1 : 32	0·0007	+	0·0004	+
1 : 64	0·0002	(+)	0·0002	(+)
1 : 128	0·0000	−	0·0000	−

* For technical reasons the dilution 1 : 2 was omitted.

† Calculated from counts of the precipitated IgG, counts of the culture medium and data of the lysine pool.

‡ The intensity of the autoradiographic image is graded from: − = negative; (+) = just visible; + = clearly visible; to +++++ = very dark.

The results of the culture experiments carried out with incubation periods of up to 6 hours show that the amount of IgG synthesized during this period is proportional to time in a sample of $0{\cdot}3\times10^8$ cells. In a sample with twice that number of cells the initial rate of synthesis is not maintained (Fig. 1). Pre-incubation of the cells with a non-radioactive medium for 3 and 6 hours shows that after these periods the IgG-synthesizing capacity of the cells has decreased (Fig. 1).

A comparison of the gradation of the autoradiographic line with the calculated amount of IgG precipitated is presented in Table 2. It is shown that the concentrated culture fluid, as routinely used in the immunoelectrophoretic analysis, gives a very distinct line, which corresponds to 0·0120 and 0·0135 μg IgG for the two spleens. The limit of visibility is reached at a dilution of approximately 1 : 64, which corresponds to 0·0002 μg IgG.

DISCUSSION

The calculations concerning the synthesis of IgG are only valid if it is assumed that the equilibrium between the extracellular [^{14}C]lysine and the intracellular pool was established rapidly and that the amino acids from both pools were incorporated without discrimination. With these conditions only the minimum amount of synthesized IgG could be calculated.

The results of this study show that isolated spleen cells synthesize about 1·25 μg IgG per 10^8 lymphoid cells during the initial 6 hours of incubation. This is an average synthesis of 1 μg/hour/g wet weight, assuming that 5×10^8 lymphoid cells weigh 1g (Nossal and Mäkelä, 1962). A comparable value is the synthesis of 0·7 μg γ-globulin/hour/g wet weight, by spleen tissue fragments of unstimulated rabbits (Thorbecke, 1960). *In vitro* studies with spleen or lymphoid cells obtained from stimulated animals show at least a ten times higher yield of immunoglobulins or antibodies (Steiner and Anker, 1956; Stavitsky and Wolf, 1958; Askonas and Humphrey, 1958; Thorbecke, 1960; Vaughan, Dutton, Dutton, George and Marston, 1960; Helmreich, Kern and Eisen, 1961).

The number of cells engaged in IgG synthesis may be estimated at 1 in 800 using the finding of Nossal and Mäkelä (1962), that a single cell synthesizes *in vitro* 1×10^{-11} g antibody per 6 hours. This figure is in the same order of magnitude as the number of antibody-producing cells found with the agar plaque technique (Jerne, Nordin and Henry, 1963; Friedman, 1964;) and the cluster technique (Zaalberg, 1964 and personal communication).

The initial rate of synthesis was not maintained during the 48 hours of incubation. Incubation studies, lasting less than 6 hours, and experiments in which the samples were pre-incubated before a radioactive medium was added, showed that after 3 hours of incubation about 25 per cent, and after 6 hours about 10 per cent, of the initial IgG synthesizing rate remained. It seems very probable that our experimental conditions did not ensure a sufficiently good environment for the cells to maintain active protein synthesis after this period. In the studies of Michaelides and Coons (1963), lymphoid cells preserved their antibody-synthesizing capacity for several weeks. The absence of serum proteins or protein-stimulating factors, such as corticosteroids or insulin (Ambrose, 1964), in our culture medium may account for this difference. Another possibility to be considered is that the concentration of amino acids in the medium was a limiting factor for the maximal synthesis of immunoglobulins (Vaughan *et al.*, 1960). No further attempt was made to elucidate this problem.

Comparison of the autoradiographs of the immunoelectrophoretic pattern with the amount of precipitated immunoglobulin from the culture fluid gives an insight into the quantity of protein which is usually detected. These results show that for an undiluted culture fluid the amount of radioactive protein visible as a very distinct line, gradation +++, amounted to about 0·0125 μg IgG; the limit of visibility (+) is reached at approximately 0·0002 μg radioactive IgG in the precipitation line.

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