SYNTHESIS OF PLASMA MEMBRANE-ASSOCIATED AND SECRETORY IMMUNOGLOBULIN IN DIPLOID LYMPHOCYTES*

By RICHARD A. LERNER,‡ PATRICIA J. McCONAHEY, INGA JANSEN, AND FRANK J. DIXON

(From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037)

During differentiation, alterations in gene expression result in the synthesis of cell-specific polypeptides. A specialized example occurs in the immune system where not only do cells synthesize immunoglobulin (Ig),[1] but the fate of these molecules presumably changes as cells differentiate. In the resting or "G_0" lymphocyte the bulk of the Ig synthesized is inserted into the plasma membrane (M-Ig), where it presumably serves as a receptor molecule for antigen recognition (1–3). After exposure to antigen, cells destined to become antibody formers undergo numerous divisions during which they presumably differentiate into plasma cells. In contrast to the product of the G_0 lymphocyte, the bulk of the Ig synthesized by the plasma cell is secreted into the extracellular fluid (S-Ig) (4). This process of differentiation is "driven" by antigen and terminates when an amount of antibody sufficient to remove antigen is secreted (5, 6).

To approach control of this process we have studied a clone of continuously growing human diploid lymphocytes. These cells appear to be arrested in differentiation somewhere between the G_0 lymphocyte and the plasma cell, and both secrete Ig and insert it as a presumed receptor into their plasma membranes (7). A method developed to quantitate the amount of Ig in the plasma membrane and cytoplasm (C-Ig) has been utilized to study the amount and/or half disappearance time of M-Ig, C-Ig, and S-Ig in logarithmically growing and synchronized cells. After treatment of logarithmically growing cells with

inhibitors of protein synthesis, the half disappearance time for detectable M-Ig is approximately 45 min. By contrast, after 24 hr of treatment of logarithmically growing cells with actinomycin D, synthesis of M-Ig is unaffected, whereas total cellular nascent polypeptide synthesis and messenger RNA (M-RNA) synthesis are reduced by approximately 95%. When cells are synchronized in G_1 (stationary phase) without the use of metabolic inhibitors, the M-Ig is reduced by only about 25%, whereas total C-Ig is decreased by 90% of that in logarithmically growing cells. Some of these results have been presented in preliminary form (7).

Materials and Methods

Reagents and Buffers.—The nonionic detergent nonidet P-40 (NP-40) was obtained from the Shell Chemical Company (New York), cytosine arabinoside from the Upjohn Company (Kalamazoo, Mich.), actinomycin D from Mann Research Laboratories (New York), ethidium bromide from the Boots Pure Drug Co., Ltd. (Poole, England), puromycin from the Sigma Chemical Company (St. Louis, Mo.), and cyclohexamide from Calbiochem (Los Angeles, Calif.). Uridine-2-^{14}C (50 mCi/mM), uridine-5-^{3}H (20 Ci/mM), L-leucine-4,5-^{3}H (30 Ci/mM), and thymidine-2-^{14}C (50 mCi/mM) were purchased from New England Nuclear Corp. (Boston, Mass.). RSB buffer was 1.0×10^{-2} M tris (hydroxymethyl) aminomethane (Tris)-HCl, pH 7.4, containing 1.0×10^{-2} M NaCl and 1.5×10^{-3} M $MgCl_2$. STE buffer was 5.0×10^{-2} M Tris-HCl, pH 7.4, containing 1.0×10^{-3} M ethylenediaminetetraacetate (EDTA) and 1.0×10^{-1} M NaCl.

Maintenance of Cells.—Suspension cultures of the 8866 line of diploid human lymphocytes were maintained in Eagle's medium containing twice the usual concentration of vitamins and amino acids and 10% fetal calf serum on a gyratory shaker as previously described (8). Cultures seeded at a concentration of 2×10^5 cells/ml reached a constant concentration of 3.0×10^6 cells/ml in 5–6 days. Cellular exclusion of 0.05% trypan blue was used as a criterion of cell viability.

Cloning of Cells.—Clones were obtained by the method of Coffino et al., except that diploid human fibroblasts (W$_i$38) were used as a feeder layer (9).

Cell Cycle Analysis.—The various phases of the cell cycle were determined by measuring the rate of DNA synthesis and number of cells in mitosis. To determine the rate of DNA synthesis, 2-ml aliquots of medium containing cells were placed into prewarmed (37°C) tubes containing 2 μCi of thymicine-2-^{14}C. After 30 min, incorporation of radioactivity was stopped by addition of 5 vol of cold (4°C) Earle's saline. Cells were washed twice in Earle's saline, collected by centrifugation at 800 g for 5 min, and resuspended in 2 ml of cold 5% trichloroacetic acid (TCA). The TCA-insoluble material was collected onto Millipore filters (Millipore Corp., Bedford, Mass.) and radioactivity measured (8). The number of cells in mitosis was ascertained using phase-contrast microscopy.

Polysome Preparation and Determination of Nascent Polypeptide Synthesis.—5×10^7 lymphocytes were suspended in 5.0 ml of prewarmed (37°C) Eagle's medium containing $\frac{1}{100}$th the normal amount of L-leucine ($\frac{1}{100}$th medium). After 15 min, 10 μCi of L-leucine-4,5-^{3}H was added and incorporation allowed to proceed for 6 min followed by addition of 8 vol of cold (5°C) Earle's saline. After centrifugation cells were resuspended for 10 min at 4°C in 1.0 ml of RSB containing 0.5% NP-40. Nuclei were removed by centrifugation at 2000 rpm at 4°C for 10 min, and the supernatant was made 0.25% with respect to sodium deoxycholate. The polyribosomes were sedimented in 7.5–45% (w/w) sucrose velocity gradients for $3\frac{1}{4}$ hr at 24,000 rpm in the SW 25.1 rotor (Spinco Division, Beckman Instruments, Palo Alto, Calif.)

at 4°C (8). Fractions were collected through a continuous recorder (0.5 cm light path) and radioactivity determined as previously described (8).

Isolation and Characterization of M-RNA.—

*Preparation of M-RNA.—*To prepare pulse-labeled M-RNA, 5.0×10^7 cells were re-suspended in 5.0 ml of warm (37°C) media without serum and incubated for 20 min with either 10 μCi/ml of uridine-2-^{14}C or 100 μCi/ml of uridine-5-^{3}H. The incorporation of radioactivity was terminated by addition of 8 vol of cold (4°C) Earle's saline, and polyribosomes were prepared as described above. The sucrose gradient fractions which contained the poly-ribosomes (approximately the bottom 40% of the gradient) were pooled, made 1% with sodium dodecyl sulfate (SDS), 2.0×10^{-3} M EDTA, and 1.0×10^{-1} M in 2-mercaptoethanol. This method for obtaining and releasing pulse-labeled RNA from eukaryotic polyribosomes conforms to current criteria for M-RNA (10). After 30 min at 22°C, NaCl was added to a final concentration of 1.0×10^{-1} M, and RNA was precipitated by addition of 2.5 vol of 95% ethanol at -20°C. If carrier nucleic acid was needed to obtain complete precipitation of RNA, 100 μg of yeast transfer RNA (Mann Research Laboratories) was added.

In some experiments, it was desirable to inhibit the synthesis of mitochondrial RNA and precursors to ribosomal RNA during pulse labeling. Accordingly, cells were incubated with ethidium bromide (1 μg/ml) and actinomycin D (0.04 μg/ml) before addition of radioactivity. As has been reported for HeLa cells (11–14), these doses of inhibitors completely suppressed respective incorporation of radioactivity into mitochondrial and ribosomal RNA in these lymphocytes but did not affect M-RNA synthesis.[2]

*Velocity Sedimentation of RNA.—*RNA was resuspended in 0.5 ml of STE buffer and separated in 16 ml 15–30% (w/w) linear sucrose gradients at 24,000 rpm for 17 hr in the SW 27 (Spinco) rotor. Gradients were analyzed for absorbance at 260 nM using a continuous-flow cell and a Gilford recording spectrophotometer (Gilford Instrument Co., Oberlin, Ohio). Radioactivity was determined as previously described (8).

*Quantitation of Cytoplasmic Ig and M-Ig.—*Since the present investigation depended on specific determination of the amount of Ig polypeptides among an excess of other cellular polypeptides, appropriate methods had to be developed. Some of these methods have been published previously (15, 16), but for clarity certain points are emphasized here.

I. *Antigens.—*Purified IgG fragments such as Fc, κ or λ chains were pooled myeloma proteins obtained from Dr. Hans Spiegelberg of our institution and iodinated with ^{125}I to achieve approximately 6.6×10^{-3} μCi/3.1×10^{-9} g of antigen protein (17). A working solution of the test antigen was prepared by dissolving the ^{125}I trace-labeled IgG fragment to be tested in borate-bovine serum albumin (BSA-BB) buffer (0.1 M borate, pH 8.3, containing 2 mg of BSA protein/ml of buffer) to a final labeled antigen concentration of 6.25×10^{-9} g of antigen protein/ml.

II. *Antibodies.—*The antibody preparation was hyperimmune rabbit anti-IgG. However, antibody used does not have to be specific for IgG alone since cross-reacting antibody to albumin, Fc-μ, etc. will not interfere with the quantitation of IgG fragments in this assay. All sera including antibody used in this system were decomplemented at 56°C for 30 min before use. Dilutions of antibody were made in decomplemented normal serum diluted 1:10 in Spinner salts containing penicillin and streptomycin as a preservative.

Since this system is based on the competition of cold unknown antigen with antigen-^{125}I for available antibody sites, it is necessary to carry out the assay in excess antigen-^{125}I (e.g., in excess antibody small amounts of cold unknown antigen would not be detectable). We have chosen as our base line that dilution of antibody which is capable of binding 50% of the IgG-^{125}I fragment added. The detailed procedure for construction of a standard curve is as follows:

(a) The base-line control = no inhibition. 50 μl of 10% heated normal serum (NS) + 0.5 or 1 ml aliquot of diluted anti-IgG.

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[2] Lerner, R. A. Unpublished observations.

(b) Inhibitors = whole IgG molecules or IgG fragments of known concentration. 50 μl of inhibitor (diluted in 2 mg of BSA-BB in 1 ml) known to contain protein in the range of 2–1500 $\times$ 10^{-9} g plus aliquot of diluted anti-IgG milliliter are added.

(c) Tubes are mixed and incubated at 4°C for 1 hr.

(d) Take duplicate or triplicate 100-μl samples from each inhibitor plus anti-IgG aliquot above and pipette into disposable 12- $\times$ 75-mm glass tubes.

(e) Add 100 μl of IgG-^{125}I fragment containing 3.1 or 6.25 $\times$ 10^{-9} g of protein.

(f) As a control for nonspecific precipitation of IgG-^{125}I fragment, add 100 μl of 10% $\triangle$ NS + 100 μl of IgG-^{125}I fragment in duplicate or triplicate. ($\triangle$ = heated)

(g) As a control for reactivity of IgG-^{125}I fragment with an excess of anti-IgG, add 100 μl of anti-IgG diluted (1:100) + 100 μl of IgG-^{125}I fragment in duplicate.

(h) Mix and incubate at 4°C for 1 hr.

(i) Add 200 μl of appropriate $(NH_4)_2SO_4$ concentration for optimum precipitation of system under study.[3]

(j) Mix well and incubate at 4°C for 30 min.

(k) Centrifuge at 3000 rpm for 30 min.

(l) Decant supernatant and discard.

(m) Resuspend precipitate in 1 ml wash $(NH_4)_2SO_4$ which is at the same concentration as the final concentration after addition for the original precipitation.

(n) Centrifuge at 3000 rpm and repeat step l.

(o) Dissolve precipitate in 100 μl of distilled water and count tubes in well-type gamma scintillation counter.

(p) Calculations:

(1) *Per cent precipitated.*—

Per cent precipitated (corrected for nonspecific precipitation) =

$$\frac{\text{experimental precipitate counts} - \text{factor} \times \text{experimental supernatant counts} \times 100}{\text{total counts added}}$$

where factor = per cent precipitated nonspecifically $\times$ (1 + per cent precipitated nonspecifically) where per cent precipitated nonspecifically = $\dfrac{\text{counts in 10\% } \triangle \text{ NS only} \times 100}{\text{total counts added}}$

where experimental supernatant counts = total counts added − experimental precipitate counts.

(2) *Per cent inhibition.*—

Per cent inhibition =

$$\frac{\text{per cent precipitated control (no inhibition)} - \text{per cent precipitated experimental} \times 100.}{\text{per cent precipitated control (no inhibition)}}$$

(q) On semilogarithmic paper, plot per cent inhibition on the linear scale *versus* total protein inhibitor added on the logarithmic scale.

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[3] The optimum $(NH_4)_2SO_4$ concentration for the precipitation of IgG-^{125}I fragments bound to antibody is dependent on the following factors: (a) type of fragment from an individual IgG molecule (i.e. Fab', Fc, etc.); (b) species from which the IgG fragment is derived (i.e. mouse, human, etc.); (c) species in which the hyperimmune anti-IgG is prepared.

The following are optimum $(NH_4)_2SO_4$ concentrations that have been determined in this laboratory: (a) rabbit anti-human IgG + human Fab'-^{125}I, 40% $(NH_4)_2SO_4$; (b) rabbit anti-human IgG + human kappa chain-^{125}I, 40% $(NH_4)_2SO_4$; (c) rabbit anti-human IgG + human lambda chain-^{125}I, 44% $(NH_4)_2SO_4$, (d) rabbit anti-human IgG + Fc-^{125}I, 40% $(NH_4)_2SO_4$; (e) rabbit anti-mouse IgG + mouse Fab'-, kappa chain-, and Fc-^{125}I, all = 47.5%.

(3) Quantitation of cell associated IgG.—

(a) A control inhibition curve is set up (as described above) with each set of cells.

(b) Wash cell thoroughly (four times in large volume of sera-free media).

(c) Resuspend cell pellet in 0.5–1 ml diluted anti-IgG in duplicate.

(d) Incubate 1 hr at 4°C, resuspending cells gently every 15 min. Control studies are carried out to insure that in handling of cells detectable amounts of IgG fragments do not "leak" from these cells.

(e) Centrifuge at 2000 rpm for 10 min (700 g).

(f) Decant absorbed anti-IgG and save; discard cell button.

(g) Add 100-μl aliquots of absorbed anti-IgG from step e to disposable 12- $\times$ 75-mm glass tubes in duplicate or triplicate.

(h) The number of cells required to be on the linear portion of the control inhibition curve varies from 1 $\times$ 10^6 (mouse peritoneal macrophages) to 2 $\times$ 10^8 (mouse thymocytes). The quantitation of human diploid lymphocytes in culture can usually be carried out with from 5 $\times$ 10^6 to 5 $\times$ 10^7 cells/test.

In order to quantitate the inhibition achieved by unknown materials suspected of containing IgG, it is necessary to determine the inhibition achieved by inhibitors (e.g., whole IgG molecules or fragments of IgG). We use whole IgG molecules from normal serum pools as standard inhibitors due to their relative constant antigenicity and express the inhibition achieved by unknowns as equivalent to a certain amount of a standard IgG preparation. In this procedure different pools of anti-IgG may give somewhat different control inhibition curves but since the assay is based on comparative degrees of inhibition of known and unknown inhibitors, identical results are achieved with a variety of anti-IgG's as long as a single standard inhibitor is used throughout. Since different IgG-blocking preparations, even those made from serum pools, will vary antigenically, their antigenic similarity to the labeled IgG fragment used will also vary. This variation is even greater when the blocking IgG's are derived from single myeloma sera. Therefore, the amount of IgG needed to achieve 50% inhibition of any reaction will depend upon the particular blocking preparation. Thus, identical data cannot necessarily be generated using different standard inhibitors.

Quantitation of as little as 1.0 $\times$ 10^{-8} g of IgG fragments/1.0 $\times$ 10^6 cells is possible. Assay specificity and adaptability to cell surfaces and extracts were determined by studying eight different cell lines with different phenotypes for M-Ig (16). Nonspecific binding of antibody to cultured lymphocytes, and thus, overestimation of the amount of Ig in various fractions did not occur. For example, lymphocyte lines which did not have Ig on the surface or in the cytoplasm did not combine with antibody (16).

To ensure that M-Ig did not simply represent secreted Ig molecules readsorbed to the plasma membrane, phenotypic mixing studies were carried out (16). WIL$_2$ and 8866 cells which had respective phenotypes for M-Ig of κ + Fc— and κ + Fc+ were grown separately for 2 days, harvested, and resuspended for 24 hr in media collected from the other cell line (e.g., WIL$_2$ cells into 8866 media). The phenotype and amount of M-Ig remained the same for each cell line, thus adsorption of secreted molecules onto plasma membranes seemed unlikely.

All the studies reported below were carried out in 8866 cells which synthesize IgG with κ-type light chains. In this cell line there were approximately 1.0 $\times$ 10^5 molecules of κ chain/cell surface and 44 times this much in the cytoplasm of each cell (16). The κ chain:Fc fragment ratio on the surface was 2.6:1 and for the total cell was 3.9:1.

RESULTS

Half Disappearance Time of M-Ig and C-Ig in Logarithmically Growing Cells Treated with Inhibitors of Protein Synthesis.—To determine the half disappearance time of M-Ig, cells were treated with puromycin and, at intervals after

treatment, the amount of κ chain and Fc fragment present on the membrane and in the cytoplasm determined (Fig. 1).

The half disappearance times for membrane-bound κ chains and Fc fragments were each approximately 45 min (Fig. 1). The equal rate of disappearance was reflected in the constant κ:Fc ratio on the membrane throughout the study (insert, Fig. 1). The initial half disappearance time for cytoplasmic κ chains
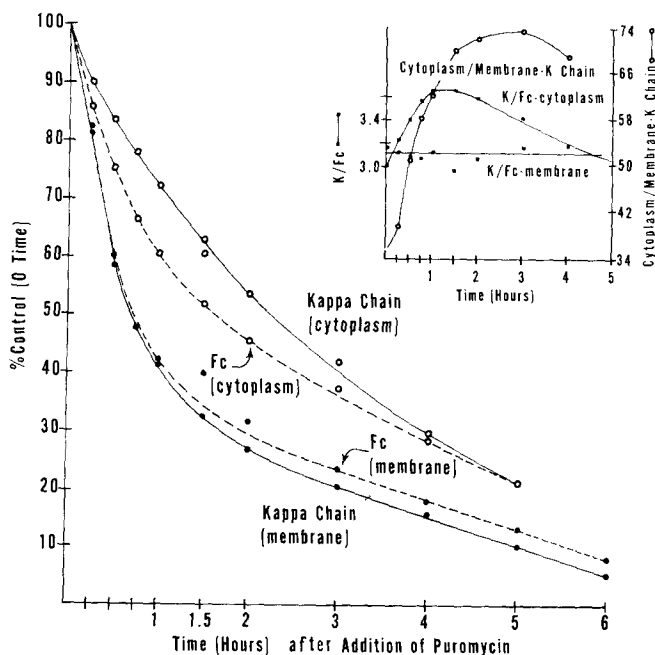

FIG. 1. The amount of M-Ig and C-Ig was determined for a culture of logarithmically growing cells (0 time), and then puromycin was added to a final concentration of 50 μg/ml. At intervals after addition of puromycin, M-Ig and C-Ig were determined.

O—O, κ chain cytoplasm; O- -O, Fc-γ fragment cytoplasm; ●—●, κ chain membrane; ●- -●, Fc-γ fragment membrane; (Insert) O—O, cytoplasmic:membrane κ chain ratio; ■, κ chain:Fc-γ fragment ratio in cytoplasm or membrane.

and Fc fragments was approximately 2 and 1.5 hr, respectively. The faster rate of disappearance for M-Ig than C-Ig was illustrated by the approximate 2-fold increase in the cytoplasm:membrane κ chain ratio in the 1st hr after puromycin treatment (insert, Fig. 1). In contrast to M-Ig κ and Fc fragments, initial rates of disappearance of cytoplasmic κ chains and Fc fragments differed from each other. This difference was shown by the increase in the κ:Fc ratio during the 1st hour after treatment with puromycin followed by a gradual return to control ratios after 5 hr (insert, Fig. 1). To ensure that the rapid disappearance of M-Ig was not an artifact caused by puromycin, the studies were repeated

with another inhibitor of protein synthesis, cyclohexamide (50 μg/ml), and similar results were obtained.

The fate of the C-Ig after treatment of cells with puromycin was determined by simultaneously measuring its accumulation in the media and disappearance from the cytoplasm. As can be seen (Fig. 2) there was almost quantitative recovery from the media of Ig lost from the cytoplasm after treatment of cells with puromycin. The fate of M-Ig cannot be established in a similar fashion, since it represents only approximately 2% of the total cellular Ig in 8866 cells.
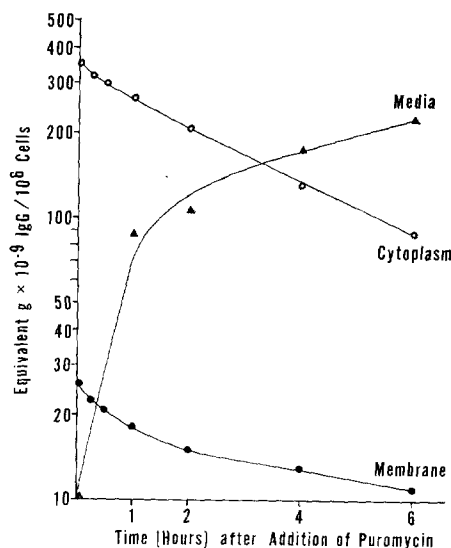

FIG. 2. Logarithmically growing cells were washed three times in Earle's salts and suspended in fresh prewarmed (37°C) media which contained puromycin (50 μg/ml) and the amount of Ig lost from the membrane or cytoplasm and that which appeared in the media determined at intervals.

O—O, C-Ig; ●—●, M-Ig; ▲—▲, Ig in the media.

To evaluate what per cent of Ig which appeared in the culture media could come from M-Ig turnover, cells were incubated in media without serum for 4 hr and the total Ig which accumulated in the media measured. 2.25×10^{-7} g of IgG equivalents appeared in the media in 4 hr for each 1.0×10^6 cells in culture; 1.0×10^6 cells had a total of 2.6×10^{-8} g of IgG equivalents on their surfaces. Using a half disappearance time for M-Ig of 45 min, approximately 6.9×10^{-8} g could appear in the media/1.0×10^6 cells in 4 hr, which would account for only approximately 3% of the total.

Amount and Half Disappearance Time of M-Ig and C-Ig in G_1 Cells.—It was of interest to determine if the amount and half disappearance time for M-Ig differered in growing and stationary phase cells. We have previously

shown that stationary phase diploid lymphocytes were arrested in the G_1 phase of the cell cycle and did not secrete Ig (8). In the present experiment, 5 days after seeding (2.0 × 10^5/ml), cells reached a maximum density of approximately 3.0 × 10^6 cells/ml, and by 8 days the rate of DNA synthesis in the culture was only 2% of maximum in logarithmically growing cells. When cells were obtained from this stationary phase culture and resuspended in fresh media, there was an 8 hr lag followed by a wave of DNA synthesis and then mitosis, showing that in these experiments stationary phase lymphocytes were arrested in the G_1 phase of the cell cycle. The amount of M-Ig and C-Ig in
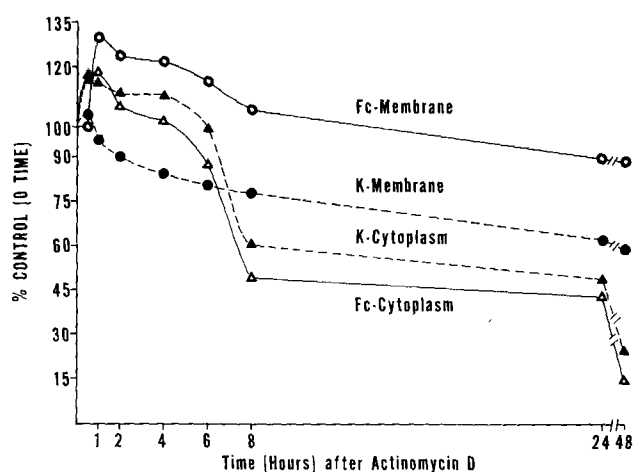

Fig. 3. The amount of M-Ig and C-Ig was determined for a culture of logarithmically growing cells (0 time), and then actinomycin D was added to a final concentration of 5 μg/ml. At intervals cell viability and the amount of M-Ig and C-Ig were determined.

●--●, κ chain membrane; ○—○, Fc-γ fragment membrane; ▲—▲, κ chain cytoplasm; △—△, Fc-γ fragment cytoplasm.

stationary phase cells was approximately 70 and 10%, respectively, of that in logarithmically growing cells, and the half disappearance of M-Ig after treatment of cells with puromycin was again 45 min.

Half Disappearance Time of M-Ig in Logarithmically Growing Cells Treated with Actinomycin D.—To determine the half disappearance time of M-Ig after treatment of cells with an inhibitor of transcription of RNA, logarithmically growing cells were treated with actinomycin D (5 μg/ml) and membrane and cytoplasmic κ chains and Fc fragments measured at intervals after drug treatment. Within the 1st hour, membrane and cytoplasmic κ chains and Fc fragments increased above control values (Fig. 3). This is consistent with our previous report that an early effect of actinomycin D in cultured lymphocytes was to increase the rate of total Ig synthesis (8). During the next 7 hr, the

amount of κ chain and Fc fragment in the membrane decreased only approximately 20% of the maximum level and then declined more slowly for the next 40 hr (Fig. 3).

In contrast to M-Ig, C-Ig declined little during the first 4 hr, rapidly in the next 4 hr to about 50% of control, then the rate changed and they decreased by
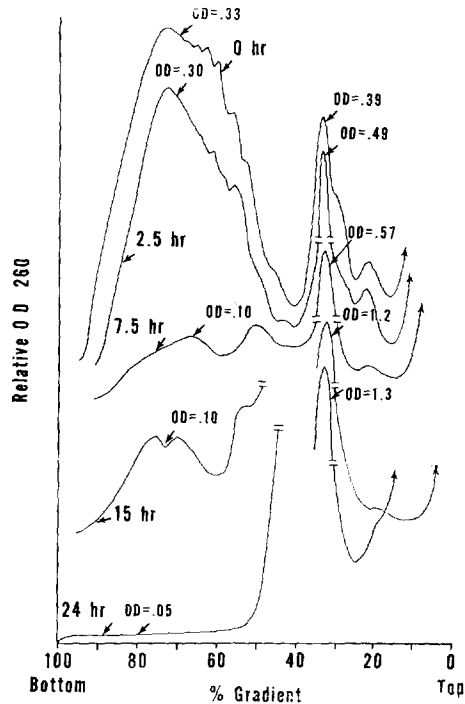

FIG. 4. Polyribosomes were prepared from 5.0 × 10⁷ logarithmically growing cells and sedimented through 7.5–45% w/w linear sucrose gradients as described in Materials and Methods (0 hr). Actinomycin D (5 μg/ml) was added, and at intervals after addition of the inhibitor, polyribosomes were prepared. The absolute OD 260 and time after addition of actinomycin D for each profile are indicated by arrows.

only an additional 20% over the next 42 hr (Fig. 3). During this time >95% of the cells remained viable.

Since one possible explanation for the above results was that the M-RNA which coded for M-Ig was long-lived, it was of interest to study the half-life of total cellular M-RNA in cultures of our lymphocytes. Accordingly, polyribosome profiles and total nascent polypeptide synthesis were studied after treatment of cells with actinomycin D (5 μg/ml). By 24 hr, the OD260 of polyribosomes had decreased to approximately 15% of control and the amount of 74S monosomes increased over 3-fold (Fig. 4). The monosome to polysome

ratio increased from approximately 1:26 in cells 24 hr after treatment with actinomycin D. During the first 15 hr after treatment, the polyribosome profile shifted from monophasic to biphasic (Fig. 4). A similar shift in polyribosome profile is seen when logarithmically growing cells are synchronized in G_1 (8). The amount of nascent polypeptide synthesis decreased with an average half time of 4 hr to 4% of control in 24 hr (Fig. 5). If the differences in the disappearance of M-Ig and C-Ig are due to cessation of synthesis of their respective M-RNA's, these results would suggest that in lymphocytes, the
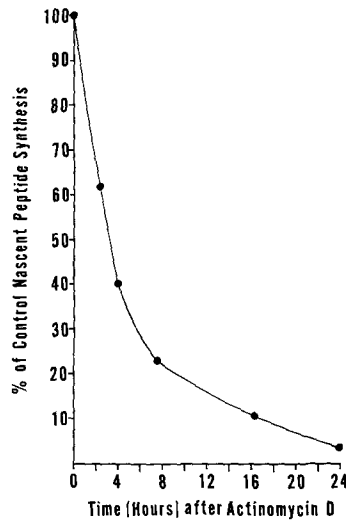

FIG. 5. To determine each point on the curve, a culture of logarithmically growing cells was divided into two. One received actinomycin D (5 μg/ml) for the interval indicated, and the other was untreated. Each culture was pulse labeled with leucine-4,5-^{3}H, polyribosomes prepared and sedimented through 7.5–45% sucrose gradients, and fractions collected as described in Materials and Methods. Radioactivity associated with the polyribosomes (approximately bottom 40% of the gradient) was determined and expressed as a percentage of nascent peptide synthesis of the untreated culture.

M-RNA which codes for M-Ig is more long-lived than 96% of other cellular M-RNA's.

Since actinomycin D affects protein synthesis only secondarily via cessation of M-RNA formation, it was important for our studies to measure directly its effects on total cellular M-RNA synthesis in the lymphocytes studied. Cells were pulse labeled for 20 min with uridine-2-^{14}C and studied either immediately or 24 hr after addition of actinomycin D (5 μg/ml). The disperse profile of sedimentation of pulse-labeled polyribosome associated RNA after a 20 min pulse, typical of that described for eukaryotic M-RNA, was obtained (10). Clearly very little information about the disappearance of rapidly labeled M-RNA

after treatment with actinomycin D could be obtained in this way, since the precursors to ribosomal RNA were also labeled in a 20 min pulse and then "chased" in the next 24 hr into 18S and 28S ribosomal RNA which completely obscured quantitative determination of any remaining M-RNA. Therefore, advantage was taken of the fact that in cells pretreated with low doses of actinomycin D (0.04 μg/ml) and ethidium bromide (1 μg/ml), transcription of ribosomal precursor RNA and mitochondrial RNA are completely suppressed
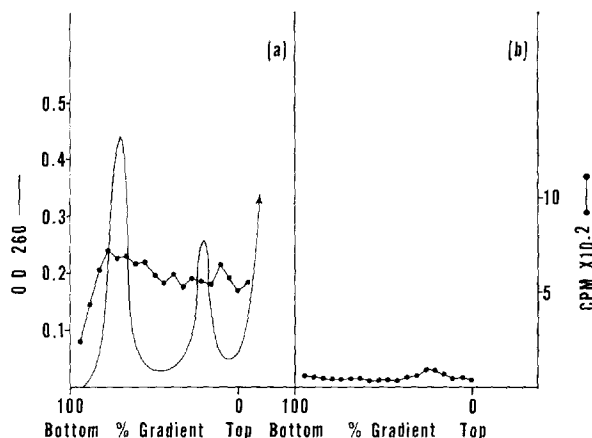

FIG. 6. 1.0 × 10^8 cells from the logarithmic phase of growth were incubated for 2 hr with actinomycin D (0.04 μg/ml) and ethidium bromide (1 μg/ml). Cells were harvested, pulse labeled for 20 min with uridine-2-^{14}C, and polyribosomes and M-RNA prepared immediately from half the culture as described in Materials and Methods. The remaining cells were resuspended in complete media containing actinomycin D (5 μg/ml) and allowed to incubate for 24 hr, and polyribosomes and M-RNA prepared. The M-RNA was sedimented through 16 ml linear 15–30% (w/w) sucrose gradients and fractions collected as described in Materials and Methods.

Panel (a), 20 min pulse-labeled M-RNA. —, OD 260 (ribosomal RNA); ●—●, cpm × 10^{-2}.

Panel (b), 20 min pulse-labeled M-RNA followed by 24 hr chase in actinomycin D (5 μg/ml). ●—●, cpm × 10^{-2}.

during a short pulse label. Therefore, after subsequent addition of large amounts of actinomycin D (5 μg/ml), one is presumably observing simply M-RNA decay uncomplicated by processing of other cytoplasmic RNA's. The results of such an experiment are illustrated in Fig. 6, panel a and b. Pretreatment of cells with low doses of actinomycin D did not suppress the synthesis of M-RNA but completely obliterated incorporation of radioactivity into ribosomal RNA even after long chase periods. The total radioactivity incorporated into M-RNA with or without pretreatment with low doses of actinomycin D (0.04 μg/ml) was almost identical. After 24 hr of treatment with 5 μg/ml of actinomycin D, only 7% of the radioactivity incorporated into M-RNA remained.

This is in good agreement with the results presented above for nascent polypeptide synthesis.

DISCUSSION

In the present investigation some features of the control of C-Ig and M-Ig have been presented. It seems clear that detectable M-Ig has a half disappearance time of approximately 45 min after inhibition of protein synthesis with puromycin and cyclohexamide. Since M-Ig disappears rapidly even in the absence of protein synthesis, it is unlikely that actinomycin D causes M-Ig to persist by terminating the synthesis of a short-lived polypeptide necessary for catabolism of M-Ig. Of course, any of the inhibitors used might affect receptors which bind M-Ig rather than synthesis of M-Ig or alter stearic arrangement of M-Ig on cell surface either of which might cause its nondetection. The fact that M-Ig persists longer after treatment with actinomycin D than approximately 90% of total cellular nascent polypeptides may offer technical advantages for its isolation.

The fates of M-Ig and S-Ig seem different in cells arrested in the stationary phase of cell growth. This study has the advantage that some of the difficulties in interpreting experiments where metabolic inhibitors are used are avoided. At this phase, M-Ig synthesis is relatively unaffected whereas, as previously shown, the synthesis of S-Ig is terminated (8, 18, 19). Also, we have demonstrated in the current work that in the stationary phase the C-Ig decreased almost 10-fold. A similar discrepancy in the behavior of C-Ig and M-Ig is seen in the present studies where metabolic inhibitors were used.

These data *in toto* suggest that maintenance of M-Ig and S-Ig may be separately controlled by the cell. Alternative explanations for the different behavior of M-Ig and S-Ig can be considered. Recently, Bloom et al. have shown that clones of cultured human lymphocytes do not exhibit genetic exclusion and can synthesize IgG and IgM simultaneously (20). Since we have not measured Fc-μ chains in these experiments, IgM molecules may have a different turnover rate than IgG molecules, thus complicating our kinetic curves of K chains. This explanation seems unlikely since our Fc-γ does not cross-react with Fc-μ,[4] and a similar complexity in kinetics is seen when either K chains or Fc-γ are studied after treatment with inhibitors. The possibility that we have two populations of cells is also unlikely since these studies were carried out with a clone of cells.

Since the amount of C-Ig is approximately 40-fold that of M-Ig, and as yet there are no markers to distinguish between S-Ig and M-Ig, the catabolic fate of M-Ig or the per cent of contribution of the C-Ig to M-Ig are difficult to establish. One would like to isolate these molecules and do pulse-chase experiments, but it is not yet possible. However, from the studies in stationary phase

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[4] Spiegelberg, H. Personal communication.

cells where synthesis of M-Ig is relatively unaffected, whereas the C-Ig decreases to almost 10%, one can speculate that at least 90% of the Ig in cells during logarithmic growth is S-Ig and is not necessary for saturation of M-Ig sites. This might be expected since in vivo the bulk of lymphocytes are in the G_c phase and synthesize relatively little protein but should presumably maintain antigen receptors.

It is impossible to determine exactly what relationship may exist between the stage of differentiation of the diploid lymphocytes in continuous culture and lymphoid cells after induction of an immune response in vivo. Morphologically, the cultured diploid lymphocytes are relatively undifferentiated having little endoplasmic reticulum and few lysosomes.[5] In this respect they resemble the G_0 lymphocyte. The "stippled" distribution of Ig on the surface of our cultured lymphocytes as studied by immunofluorescence is similar to that seen in some human peripheral blood mononuclear leukocytes stained suspension.[6] As continuous cultures capable of responding to specific antigens become available it may be possible to drive the cultured cells to differentiate, and we can then answer with certainty some of these unresolved problems.

SUMMARY

The half disappearance time for detectable plasma membrane-associated and cytoplasmic immunoglobulin after treatment of continuously growing diploid lymphocytes with inhibitors of protein and RNA synthesis was studied. Also, the amount of plasma membrane-associated and cytoplasmic immunoglobulin of synchronized cells in the G_1 phase of the cell cycle has been studied. Plasma membrane-associated immunoglobulin has a half disappearance time of 45 min after inhibition of protein synthesis. By contrast, after treatment of cells with actinomycin D for 24 hr, plasma membrane-associated immunoglobulin remains relatively unchanged whereas cytoplasmic immunoglobulin decreased by almost 90%. In the G_1 phase of the cell cycle, plasma membrane-associated immunoglobulin and cytoplasmic immunoglobulin were 70 and 10%, respectively, of that in logarithmically growing cells, and the half disappearance of M-Ig after treatment of cells with puromycin was again 45 min. *In toto*, these results suggest that perhaps secreted and plasma membrane-associated immunoglobulin may be separately controlled by the cells.

BIBLIOGRAPHY

1. Wigzell, H., and B. Andersson. 1969. Cell separation on antigen-coated columns. Elimination of high rate antibody-forming cells and immunological memory cells. *J. Exp. Med.* **129:**23.
2. Sell, S., and R. G. H. Gell. 1965. Studies on rabbit lymphocytes in vitro. I. stim-

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[5] Lerner, R. A., and J. D. Feldman. Unpublished observations.
[6] Dixon, F. J., and P. J. McConahey. Unpublished observations.

ulation of blast transformation with an antiallotype serum. *J. Exp. Med.* **122:** 423.

3. Pernis, B., L. Forni, and L. Amante. 1970. Immunoglobulin spots on the surface of rabbit lymphocytes. *J. Exp. Med.* **132:**1001.

4. Paraskevas, F., L. Sho-Tone, and L. G. Israels. 1970. Absence of γ-globulin receptors on mouse plasmacytoma cells. *Nature (London).* **227:**395.

5. Uhr, J. W., and G. Möller. 1968. Regulatory effect of antibody on the immune response. *Advan. Immunol.* **8:**81.

6. Dixon, F. J., H. Jacot-Guillarmod, and P. J. McConahey. 1967. The effect of passively administered antibody on antibody synthesis. *J. Exp. Med.* **125:**1119.

7. McConahey, P. J., R. A. Lerner, and F. J. Dixon. 1971. Quantitation of plasma membrane and cytoplasmic Ig of human diploid lymphocyte clones. *Fed. Proc.* **30:**588.

8. Lerner, R. A., and L. D. Hodge. 1971. Gene expression in synchronized lymphocytes: studies on the control of synthesis of immunoglobulin polypeptides. *J. Cell. Physiol.* **77:**265.

9. Coffino, P., R. Laskow, and M. D. Scharff. 1970. Immunoglobulin production: method for quantitatively detecting variant myeloma cells. *Science (Washington).* **167:**186.

10. Darnell, J. E. 1968. Ribonucleic acids from animal cells. *Bacteriol. Rev.* **32:**262.

11. Zylber, E., C. Vesco, and S. Penman. 1969. Selective inhibition of the synthesis of mitochondria associated RNA by ethidium bromide. *J. Mol. Biol.* **44:**195.

12. Perry, R. P. 1964. Role of the nucleolus in ribonucleic acid metabolism and other cellular processes. *Nat. Cancer Inst. Monogr.* **14:**73.

13. Dubin, D. T. 1967. The effect of actinomycin on the synthesis of mitochondrial RNA in hamster cells. *Biochem. Biophys. Res. Commun.* **29:**655.

14. Penman, S., C. Vesco, and M. Penman. 1968. Localization and kinetics of formation of nuclear heterodisperse RNA, cytoplasmic heterodisperse RNA and polyribosome-associated messenger RNA in Hela cells. *J. Mol. Biol.* **34:**49.

15. Smith, R. J., Longmire, R. T. Reid and R. S. Farr. 1970. The measurement of immunoglobulin association with human peripheral lymphocytes. *J. Immunol.* **104:**367.

16. Lerner, R. A., P. J. McConahey, and F. Dixon. 1971. Quantitative aspects of plasma membrane associated immunoglobulin in clones of diploid human lymphocytes. *Science (Washington).* **173:**60.

17. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* **29:**185.

18. Buell, D. N., and J. L. Fahey. 1969. Limited periods of gene expression in immunoglobulin synthesizing cells. *Science (Washington).* **164:**1524.

19. Takahashi, M., Y. Yagi, G. E. Moore, and D. Pressman. 1969. Immunoglobulin production in synchronized cultures of human hematopoietic cell lines. I. Variation of cellular immunoglobulin level with the generation cycle. *J. Immunol.* **103:**834.

20. Bloom, A. D., K. W. Choi, and B. J. Lamb. 1971. Immunoglobulin production by human lymphocytoid lines and clones: absence of genic exclusion. *Science (Washington).* **172:**382.