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Early Changes in Immunoglobulin M Synthesis after Mitogenic Stimulation of Bone Marrow Derived Lymphocytes†

Fritz Melchers* and Jan Andersson‡

ABSTRACT: Small resting B-lymphocytes synthesize 10% of their total cellular pool of immunoglobulin M (IgM) molecules within 2–4 hr. They release these IgM molecules from the cells into the supernatant medium with a half-disappearance time between 20 and 40 hr, mainly as 7–8 subunit IgM_s. Bacterial lipopolysaccharides (LPS) stimulate small resting B-lymphocytes within the first hour to a two- to threefold increased rate of IgM synthesis. This stimulation is mitogen-dose dependent and can also be observed with certain batches of fetal calf serum and with the purified protein derivative of tuberculin, two other B-cell mitogens. IgM molecules synthesized with this increased rate are actively secreted from the cells with a median disappearance time of 2–4 hr, mainly as 19S IgM pentamers. The initial change in the rate and type of IgM synthesis after mitogenic stimulation can also be observed in the presence of 5 μg/ml of actinomycin D. IgM synthesis, which is more sensitive to inhibition by actinomycin D than the sum of all cellular protein syntheses in small unstimulated B-lymphocytes, is rendered more resistant to this inhibitor immediately after mitogenic stimulation. Stimulation of small, resting B-lymphocytes by mitogens, in the presence or absence of actinomycin D, leads to a redistribution of ribosomes from monoribosomes to polyribosomes within the first hour after stimulation. It appears, therefore, that stimulation of small, resting B-lymphocytes by mitogens leads to stabilization of RNA synthesis-dependent components of IgM synthesis, such as messenger RNA, from degradation through the formation of polyribosomes and re-programs IgM synthesis from a synthesis of surface membrane bound receptor IgM to a synthesis of actively secreted IgM.

Thymus-derived (T) and bone marrow derived (B) lymphocytes[1] cooperate in the immune response against most antigens (Miller and Mitchell, 1969). B-Lymphocytes produce and secrete immunoglobulins (Ig). Small, resting B-lymphocytes contain Ig, mainly of IgM class, in their surface membrane (Greaves and Hogg, 1971). These membrane-bound Ig molecules are thought to serve as receptors for antigen. Binding of antigen initiates reactions in the resting, so-called antigen-sensitive cells which lead to proliferation of clones of lymphocytes in which B-cells differentiate to Ig-secreting plasma cells.

Mitogens can mimic the action of antigen on lymphocytes. Bacterial lipopolysaccharide (LPS), the purified protein derivative of tuberculin, or certain batches of fetal calf serum (see articles in Möller, 1972) stimulate B-lymphocytes to proliferate and to differentiate into Ig-secreting plasma cells. While an antigenic determinant stimulates

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[1] Abbreviations used are: T-lymphocytes, T-cells, thymus-derived lymphocytes; B-lymphocytes, B-cells, bone marrow derived lymphocytes; IgM, immunoglobulin M; LPS, bacterial lipopolysaccharide from Gram-negative bacteria; PFC, plaque-forming cells.

only a very small portion of all lymphocytes (at best 0.02% (Ada, 1970)), mitogens stimulate between 20 and 70% of them. Mitogen-stimulated lymphocytes can thus be used to study biochemical changes in the mode of Ig synthesis which occur during proliferation and differentiation.

Small, resting bone marrow derived (B) lymphocytes synthesize around 250–500 immunoglobulin M (IgM) 7–8S subunit molecules per hour per cell. The main cellular pool of these IgM molecules is the surface membrane. From the surface membrane the IgM molecules are shed into the extracellular fluid with a median disappearance time between 20 and 80 hr, as 7–8S subunit IgM's. These IgM molecules contain the "core" sugars, glucosamine and mannose, but not the "branch" sugars, galactose and fucose, in carbohydrate moieties attached to the μ-heavy chains (Melchers and Andersson, 1973; Melchers *et al.*, 1974; Andersson *et al.*, 1974).

After exposure to mitogens, B-cells develop which actively secrete IgM molecules as 19S pentamers, containing not only the "core," but also the "branch" sugars (Andersson and Melchers, 1973; Melchers and Andersson, 1974). The median disappearance time of actively secreted IgM molecules has been measured to be around 4 hr (Melchers and Andersson, 1973, 1974). After activation, IgM synthesis and secretion increase *selectively* over synthesis and secretion of other proteins and glycoproteins in the B-cells (Melchers and Andersson, 1973, 1974; Andersson and Melchers, 1973). While IgM synthesis is more sensitive to actinomycin D inhibition than all other cellular protein syntheses in small, resting lymphocytes, it is more resistant in large plasma cell-like, mitogen-activated lymphocytes (Melchers *et al.*, 1974; Andersson *et al.*, 1974; Melchers and Andersson, 1974).

These changes in the biochemical parameters of IgM synthesis after mitogenic stimulation of B-lymphocytes have previously been measured 1–3 days after stimulation. Analyses of biochemical changes in B-cells within the first hours of mitogenic stimulation have previously been obscured by the presence of a small number (~1%) of large, antibody-secreted plasma cells in normal, experimentally unstimulated spleen cell suspensions. We now purify small resting lymphocytes from contaminating large, antibody-secreting cells by velocity sedimentation fractionation (Melchers *et al.*, 1974; Andersson *et al.*, 1974; Miller and Phillips, 1969). We use B-lymphocytes from "nude" mice. These nude mice, as a consequence of a genetic defect, lack T-cells (Pantelouris, 1968). Using these purified small lymphocytes as targets for mitogenic stimulation we are now able to detect changes in IgM synthesis within the first hour after mitogenic stimulation.

Materials and Methods

Spleen cell suspensions were prepared from athymic "nude" mice (normal unstimulated B-cells) as described (Andersson and Melchers, 1973). Large cells including high rate IgM synthesizing and secreting background plaque-forming cells were removed from small, resting B-cells in these cell suspensions by velocity sedimentation over ficoll gradients (Melchers *et al.*, 1974; Andersson *et al.*, 1974; Miller and Phillips, 1969). Between 30 and 40% of these cells were Ig positive as determined by staining for surface-bound Ig with fluorescent anti-Ig antibodies. Ficoll had no mitogenic activity on the small lymphocyte population. Lymphocytes from the ductus thoracicus of nude mice were obtained as described (Sprent, 1973) and kindly donated by Dr. J. Sprent at our Institute. Between 92 and 96% of these cells were Ig-positive in fluorescent staining for surface-bound Ig molecules. Cell viabilities were determined by trypan blue exclusion (Hoskins *et al.*, 1956). Lipopolysaccharide from *Salmonella abortus equii* (preparations ED TEN 18735 and S429-18735) were kindly given to us by Drs. Chris Galanos and Otto Lüderitz, Max-Planck-Institut für Immunbiologie, Freiburg, Germany. The purified protein derivative of tuberculin, batch RT27, was from Statens Seruminstitut, Copenhagen, Denmark (1 mg containing 50,000 T.U. units). Fetal calf serum batch 42123 from Flow Laboratories, Irvine, Scotland, was used. Labeling of cells with radioactive leucine (L-[4,5-^{3}H]leucine, batch 42, 51 Ci/mmol–66 μCi/ml; L-[U-^{14}C]leucine, batch 89, 342 mCi/mmol, 10 μCi/ml, both from the Radiochemical Centre, Amersham, U.K.) in Eagle's medium minus leucine (Gibco), the chase of radioactively labeled cells in unlabeled, complete Eagle's medium (Flow Laboratories), separation of the cells from the supernatant media, and lysis of the cells by the nonionic detergent Nonidet P40 (NP40, Shell Chemical Co., Zürich, Switzerland) were done as described in previous publications (Melchers and Andersson, 1973, 1974; Andersson *et al.*, 1974; Andersson and Melchers, 1973), except that serum-free media were used unless specified otherwise in the Results section. Incorporation of radioactive leucine into acid-precipitable total cellular and secreted protein was determined as described (Melchers and Andersson, 1973). A detailed description of the sandwich technique of immunoprecipitation used for the quantitation of synthesized and secreted radioactive Ig as well as the analyses of the specificities of the antisera employed in this work have been given elsewhere (Melchers and Andersson, 1973, 1974; Andersson *et al.*, 1974; Andersson and Melchers, 1973). We thank Dr. F. Franek, Czechoslovakian Academy of Sciences, Prague, Czechoslovakia, for the preparation of the pig anti-rabbit Ig antiserum used in these sandwich precipitation tests. Radioactive IgM synthesized and released by the cells was assayed for its size and polypeptide chain composition by polyacrylamide gel analysis on 2.5% cross-linked composite gels containing 0.5% agarose using radioactive sandwich precipitates dissociated in 8 M urea containing 0.1% sodium dodecyl sulfate (Andersson *et al.*, 1974; Peacock and Dingman, 1968; Choules and Zimm, 1965) for size, and on 7.5% cross-linked gels using radioactive sandwich precipitates dissociated and reduced in 8 M urea–0.1% sodium dodecyl sulfate–10^{-3} M dithiothreitol (Andersson *et al.*, 1974; Parkhouse and Melchers, 1971), for polypeptide composition. Simultaneous determinations of ^{14}C and ^{3}H radioactivities in samples were done in a Packard scintillation counter as described previously (Melchers and Andersson, 1973). Actinomycin D (Sigma Chemical Co., St. Louis, Mo.) was dissolved in ethanol at 1 mg/ml and the solution kept cold and dark until use. For the analysis of the ribosomal pattern 2–5 $\times$ 10^7 cells in 10 ml of Eagle's medium were treated as described in the Results section and then lysed in 0.5–1 ml of 0.01 M NaCl–0.003 M $MgCl_2$–0.01 M Tris-HCl (pH 7.4) containing rabbit liver supernatant as a ribonuclease inhibitor (Blobel and van Potter, 1966), 10^{-2} M cycloheximide to freeze growing polypeptide chains on ribosomes, and 0.5% NP40 as membrane-dissolving detergent. After 10 min at 0° the lysate was spun at 4° for 5 min at 5000 rpm in the Sorvall RS-2B centrifuge using the SS34 rotor. The resulting supernatant lysate was layered on a convex exponential sucrose gradient (Melchers, 1972) and spun at 4° for 30

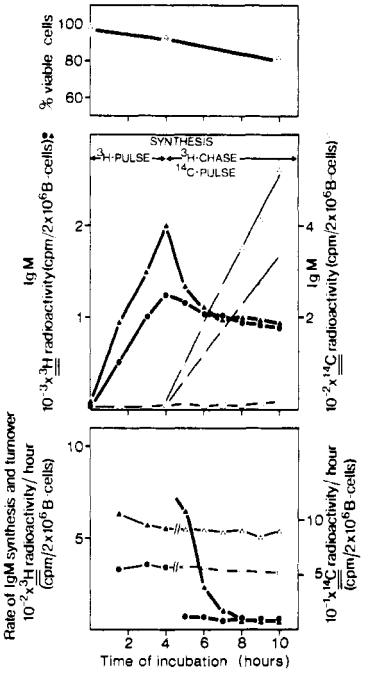

FIGURE 1: Incorporation of radioactive leucine into small lymphocytes from spleens of nude mice in the presence (▲,△) and absence (●,○) of LPS. After a pulse of 4 hr in [^{3}H]leucine (▲, ●) cells were transferred into medium containing [^{14}C]leucine (△, ○). Medium with radioactive leucine, but without cells (□), was analyzed as a control. Viabilities of cells in the presence (△) and absence (○) of LPS were determined at the beginning and after 4 and 10 hr (top). Radioactive IgM inside the cells was determined by serological precipitations (middle). Details are given in the Materials and Methods section and in the text. At the bottom part of the figure, rates of IgM synthesis respective to disappearance from the cells are given. It is evident that during the chase in [^{14}C]leucine medium a drastic change in the rate of disappearance of [^{3}H]leucine-labeled IgM is detectable in LPS-stimulated cells from a rapid to a slower rate.

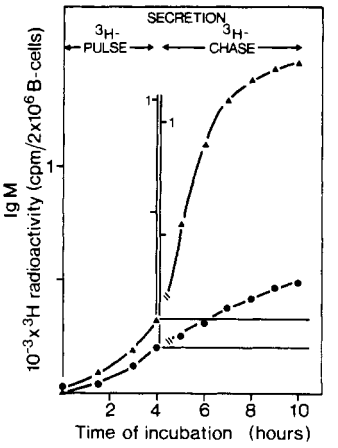

FIGURE 2: Appearance of [^{3}H]leucine-labeled IgM in the supernatant medium of small lymphocytes from the spleens of nude mice in the presence (▲) and absence (●) of LPS. Details are given in the Materials and Methods section and throughout the text. Since after 4 hr cells were transferred into new medium not containing [^{3}H]leucine, the time of release of [^{3}H]leucine-labeled IgM and the amount of labeled IgM released from the cells during the chase period are added to the time of the [^{3}H]leucine pulse (4 hr) and to the amount of labeled IgM released from the cells during the pulse period. Thus, the inserted graphs have their starting points at 4 hr of incubation time at the amount of [^{3}H]leucine-labeled IgM released up to that time.

min at 50,000 rpm in the Beckman Ultracentrifuge L-50, without braking, using the swinging bucket rotor SW-56. The gradients were recorded for absorptions at 260 nm.

Results

Rate of IgM Synthesis and Release in Small Lymphocytes before and after Stimulation with the Mitogen Lipopolysaccharide

IgM Synthesis. Purified small, resting lymphocytes from spleens of nude mice were cultured in medium containing [^{3}H]leucine in the absence or presence of 20 μg/ml of lipopolysaccharide (LPS). Synthesis of IgM was monitored by the incorporation of ^{3}H radioactivity into intracellular protein precipitable by IgM-specific antisera (Figure 1, ^{3}H pulse period). It can be seen that the rate of intracellular accumulation of IgM is higher in LPS-stimulated than in the same number of unstimulated B-cells. Approximately one-tenth of the radioactive IgM synthesized during the 4-hr labeling period appeared at that time in the extracellular medium of either unstimulated or LPS-stimulated cells (Figure 2). Since release of [^{3}H]leucine-labeled IgM within 4 hr is so low compared to the amount of intracellular labeled IgM, and since it is approximately the same for unstimulated and LPS-stimulated cells during that time, we will use the term "IgM synthesis" for what should more precisely be called "intracellular accumulation of IgM."

After 4 hr the cells were removed by centrifugation from the medium containing [^{3}H]leucine and were placed into medium containing [^{14}C]leucine. For the following 6 hr the rate of IgM synthesis, now measured as ^{14}C radioactivity in serologically precipitable IgM, was again higher in LPS-stimulated cells than in unstimulated cells (Figure 1, ^{14}C pulse, ^{3}H chase period).

The rate of incorporation of [^{3}H]leucine into cellular protein and into IgM recovered in the cells and in the extracellular medium was constant for the 4-hr labeling period (Figure 1, lower part). Incorporation of [^{3}H]leucine was stopped completely by the chase in [^{14}C]leucine medium.

Release of IgM. Disappearance of [^{3}H]leucine-labeled IgM from the cells, synthesized in the first 4 hr of the experiment, can be followed within the next 6 hr, during the [^{14}C]leucine labeling period. From unstimulated cells, [^{3}H]leucine labeled IgM disappeared with a median disappearance time of 20–40 hr (Figure 1, ^{14}C pulse/^{3}H chase period measured for 6 hr). This slow disappearance is in agreement with earlier results on the rate of turnover of IgM in unstimulated small lymphocytes (Melchers *et al.*, 1974; Andersson *et al.*, 1974). ^{3}H-Labeled IgM disappeared from LPS-stimulated cells in two phases. The initial phase of disappearance was rapid, with an estimated half-time of 2–4 hr. It was followed by a slow phase which in total amount of IgM and in its half-time of disappearance (20–40 hr) was very similar to that seen in the same number of unstimulated cells. Cell viabilities during the 10-hr incubation period dropped from 97 to 99% at the beginning to around 80% at 10 hr in both unstimulated and LPS-stimulated cultures.

Appearance of IgM Molecules in the Supernatant Medium. IgM molecules which disappear from unstimulated or LPS-stimulated cells can be detected in supernatant medium by precipitation with IgM-specific antisera. Kinetic studies (see Figure 1) show that [^{3}H]leucine-labeled IgM molecules appear with a slow rate in the supernatant medium of unstimulated small B-cells (Figure 2). Thus, in 10 hr between 30 and 40% of the [^{3}H]leucine-labeled IgM synthesized in the first 4 hr is released. Leucine-labeled IgM

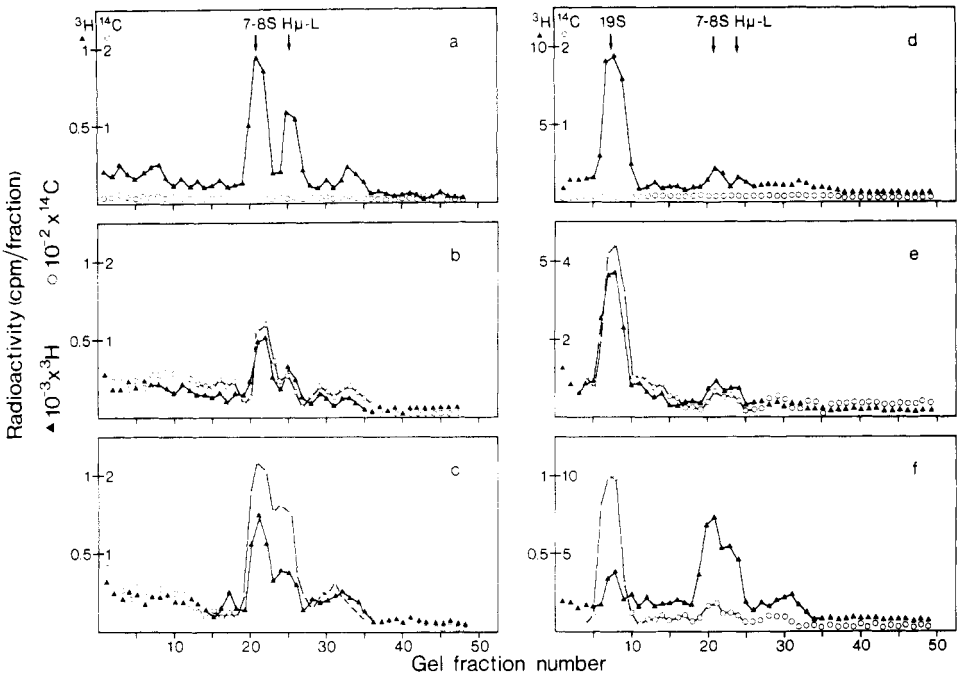

FIGURE 3: Size analysis of the leucine-labeled extracellular IgM released from: (a) unstimulated cells after 4 hr of [^{3}H]leucine labeling; (b) unstimulated cells after a subsequent 2-hr chase period in [^{14}C]leucine-containing medium; (c) unstimulated cells after a 4-hr chase period subsequent to the first 2-hr chase period in [^{14}C]leucine-containing medium; (d) LPS-stimulated cells after 4 hr of [^{3}H]leucine labeling; (e) LPS-stimulated cells after a subsequent 2-hr chase period in [^{14}C]leucine-containing medium; (f) LPS-stimulated cells after a 4-hr chase period subsequent to the first 2-hr chase period in [^{14}C]leucine-containing medium (see also Figure 1). Details are given in the text under Materials and Methods.

molecules rapidly released from LPS-stimulated cells are detected between 2 and 4 hr after stimulation. Consequently, from these B-cells, more than 50% of the labeled IgM molecules synthesized during the first 4 hr of LPS stimulation are released into the supernatant medium within the next 3 hr (Figure 2).

Size Analysis of the Released IgM. The size of radioactive IgM synthesized and released from small lymphocytes in the presence and absence of LPS was determined on composite agarose–polyacrylamide gels (Peacock and Dingman, 1968) adapted to the soluble gel procedure of Choules and Zimm (1965). Radioactive IgM serologically precipitated from the supernatant media of cells (a) after a 4-hr pulse period in [^{3}H]leucine medium, (b) after a first chase period of 2 hr in [^{14}C]leucine medium, and (c) after a second chase period of another 4 hr in new, [^{14}C]leucine medium was analyzed on the gels (Figure 3).

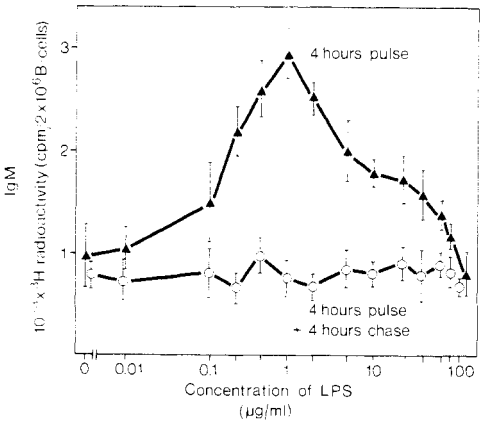

FIGURE 4: Mitogen dose dependence of the early change in the rate of IgM synthesis after addition of LPS to small, resting lymphocytes, from spleens of nude mice as measured by the incorporation of [^{3}H]leucine into serologically precipitable IgM during 4 hr of labeling (▲) and after a subsequent chase of 4 hr in nonradioactive medium (○). Bars indicate the geometric mean of four determinations. For details see the Materials and Methods section.

In agreement with earlier findings (Melchers *et al.*, 1974; Andersson *et al.*, 1974), IgM was released from the unstimulated cells during the whole pulse-chase period in the ^{3}H- as well as ^{14}C-labeled forms as 7–8S subunit IgM_s and as Hμ–L precursors (Figures 3a–c).

LPS-stimulated cells released approximately 10 times more IgM during the 4-hr pulse-chase period in [^{3}H]leucine than unstimulated cells. Of the released IgM 90% were 19S IgM pentamers, 7% IgM_s subunits, and less than 3% Hμ–L precursors (Figure 3d). During the first chase period in [^{14}C]leucine medium around 90% of the [^{3}H]leucine-labeled IgM were released again as 19S IgM pentamers. Most of the detectable [^{14}C]leucine-labeled IgM was also 19S IgM (Figure 3e). During the second chase period in new [^{14}C]leucine medium, more than 80% of the [^{3}H]leucine-labeled IgM were now 7–8S subunit IgM_s and Hμ–L precursors. Only 20% were 19S IgM pentamers. [^{14}C]Leucine-labeled IgM, however, continued to be released as 19S IgM pentamers (Figure 3f). Thus, the LPS-stimulated cells had not lost their capacity to polymerize newly synthesized IgM to 19S molecules. The pool of [^{3}H]leucine-labeled molecules, however, which could be polymerized to 19S IgM had largely been depleted during the first 2-hr chase period.

We conclude that stimulation of small, resting B-lymphocytes by the mitogen lipopolysaccharide results in an increase in the rate of IgM synthesis within the first hour after stimulation. IgM molecules are released from unstimulated small lymphocytes with a half-time of 20–40 hr as 7–8S subunit IgM_s. IgM molecules synthesized at the increased rate in LPS-stimulated cells above that in unstimulated cells are released rapidly with a half-time of 2–4 hr and are polymerized to 19S pentamers. Our results indicate

that IgM can follow two intracellular pathways: one in which it is deposited into the surface membrane as receptor Ig, turning over slowly as a 7–8S subunit, and the other in which it is actively secreted as 19S pentamer at a high rate. Similar results were also obtained with small circulating B-lymphocytes obtained from the ductus thoracicus of nude mice.

Dose Dependence of the Stimulation of IgM Synthesis. Stimulation of small resting lymphocytes to increased IgM synthesis was LPS-dose dependent. Cells were incubated for 4 hr in [^{3}H]leucine-containing medium with different doses of LPS and an aliquot of the labeled cells was assayed for their content in [^{3}H]leucine-labeled IgM. Doses between 0.2 and 5 μg/ml of LPS gave two- to threefold increases in the initial rate of IgM synthesis (Figure 4).

In another aliquot of the labeled cells the radioactive medium was then replaced by nonradioactive medium in which the labeled cells were allowed to synthesize nonlabeled IgM and release previously labeled IgM for another 4 hr. Then these labeled, chased cells were assayed for their content of [^{3}H]leucine-labeled IgM. The results (Figure 4) show that the content of labeled IgM is the same after the 4-hr pulse–4-hr chase period in cells stimulated with different doses of LPS as in cells not stimulated with the mitogen. Thus, IgM molecules synthesized at increased rates above the rate of synthesis in unstimulated cells are released rapidly, *i.e.* within the 4-hr chase period.

Increase in the Rate of IgM Synthesis Induced by Other B-Cell Mitogens. Two other B-cell mitogens, fetal calf serum (Coutinho *et al.*, 1973) and the purified protein derivative of tuberculin (Nilsson *et al.*, 1973), were tested for their capacity to induce this early increase in the rate of synthesis of IgM molecules released with a rapid rate from originally small B-cells.

Fetal calf serum as well as the purified protein derivative of tuberculin increase the rate of IgM synthesis in small, resting B-cells within the first hour after exposure to the mitogen. The increase again is dependent on the dose of mitogen, being highest for the highest concentration of the purified protein derivative of tuberculin employed (50 μg/ml) and optimal between 5 and 10% of fetal calf serum (shown in Figure 5). The optimal increase in the rate of IgM synthesis is a two- to threefold stimulation. IgM molecules synthesized at the increased rate disappear rapidly from the mitogen-stimulated cells.

We conclude that the three B-cell mitogens, LPS, purified protein derivative, and fetal calf serum, all induce an increased rate of synthesis of IgM molecules within the first hours of stimulation. These IgM molecules are rapidly released from the cells.

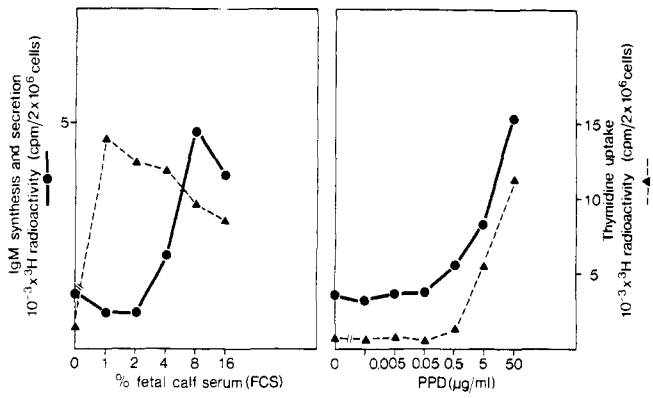

FIGURE 5: Mitogen dose dependence of the early change in the rate of IgM synthesis after addition of fetal calf serum (left) and purified protein derivative (PPD) of tuberculin (right) to small, resting lymphocytes, from spleens of nude mice as measured by the incorporation of [^{3}H]leucine into IgM during 4 hr of labeling (-●). For comparison, the mitogen dose dependent induction of DNA synthesis by these two mitogens, measured at 48 hr of stimulation, is also given (- -▲). For details see the Materials and Methods section.

IgM Synthesis in Unstimulated and LPS-Stimulated B-Lymphocytes in the Presence of Actinomycin D. Actinomycin D inhibits DNA-dependent RNA synthesis. Thus, we can test whether the early changes observed after mitogenic stimulation of small, resting B-lymphocytes occur in the absence of RNA synthesis in actinomycin D inhibited cells. Actinomycin D, at 5 μg/ml, inhibits 98% of the uptake of radioactive uridine into RNA in these cells (unpublished observations). At this concentration, actinomycin D was added 20 min prior to the start of leucine labeling and of LPS stimulation to allow saturation of uptake of the drug into the cells (Ringertz *et al.*, 1969). IgM synthesis was measured by serological determination of leucine-labeled IgM as described above. Initial rates of IgM synthesis were determined as the amount of radioactive IgM synthesized during the first hour or during the first 2 hr of labeling, in the presence or absence of actinomycin D with or without stimulation by LPS.

At 1 hr after initiation of stimulation and labeling, rates of IgM synthesis in LPS-stimulated cells were two- to threefold above those in unstimulated cells. In the presence of actinomycin D levels of IgM synthesis in both unstimulated and LPS-stimulated cells were approximately 80% of those obtained in the absence of actinomycin D (Table I).

At 2 hr after stimulation there was still a two- to threefold difference in the rates of IgM synthesis between unstimulated and LPS-stimulated cells. The presence of actinomycin D, however, had effected a decrease of the abso-

TABLE I: Rates of IgM Synthesis[a] during the First Hour and the First Two Hours after Stimulation by LPS[b] in Small Lymphocytes from Spleens of Nude Mice either Exposed or Not Exposed to Actinomycin D.[c]

	$10^{-3} \times$ cpm/2 $\times 10^6$ cells			
	0–1 hr		0–2 hr	
	− Actinomycin D	+ Actinomycin D	− Actinomycin D	+ Actinomycin D
− LPS	2.1 ± 0.4	1.9 ± 0.3	4.3 ± 0.7	2.2 ± 0.5
+ LPS	4.5 ± 0.8	3.9 ± 0.6	8.8 ± 1.0	4.6 ± 0.7

[a] "Synthesis" comprises radioactive IgM in cellular lysate plus extracellular fluid. [b] 10 μg/ml. [c] 5 μg/ml added 20 min before [^{3}H]leucine pulse and LPS stimulation (see text).

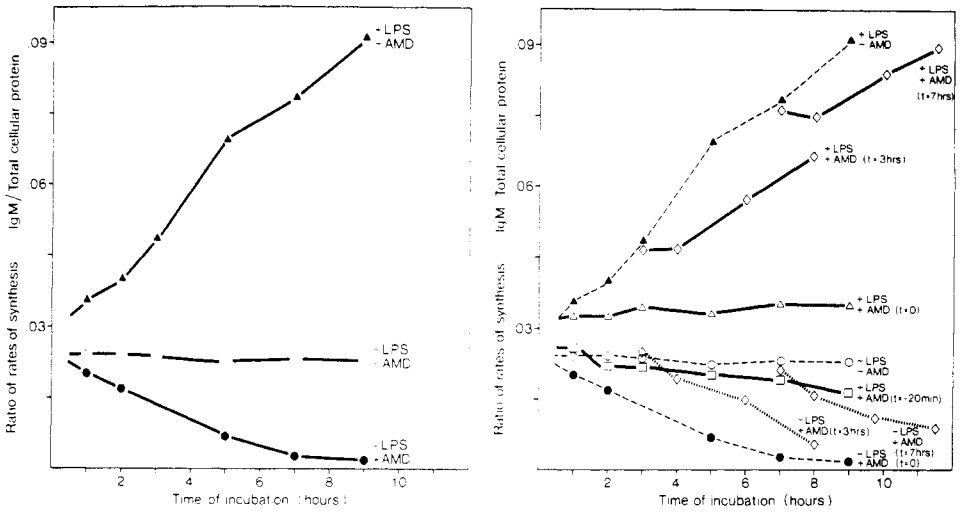

FIGURE 6: Changes in the ratio of rates of synthesis of IgM over those of all cellular proteins determined in 2-hr pulses of incorporation of radioactive leucine into small lymphocytes from spleens of nude mice in the absence or presence of actinomycin D (± AMD) and in the presence or absence of LPS (± LPS). LPS was always added at time 0.

lute rate of IgM synthesis to half of the rates in cells not exposed to actinomycin D (Table I).

We conclude that the early increase in IgM synthesis within the first hour after LPS stimulation can be observed in the absence of "de novo" RNA synthesis.

Preferential IgM Synthesis in LPS-Stimulated B-Lymphocytes. The presence of actinomycin D effects a net decrease in the rates of total protein synthesis and in IgM synthesis (see Table I) in small lymphocytes, regardless of whether LPS is added to the cells or not. If, however, rates of IgM synthesis are compared with rates of synthesis of all cellular proteins during the first 10 hr of stimulation, changes after stimulation become apparent. The ratio of the rates of IgM synthesis over those of all cellular proteins increases in the absence of actinomycin D from the first hour of stimulation with LPS (Figure 6, left). There is no change in this ratio over a 10-hr period in the absence of stimulation and in the absence of actinomycin D (Figure 6, left). These results extend earlier findings that stimulation of B-lymphocytes results in a *selective* increase in the rate of IgM synthesis over the rates of synthesis of all cellular proteins after mitogenic stimulation (Melchers and Andersson, 1973, 1974; Andersson and Melchers, 1973). We can now state that this increase is detectable within the first hour of stimulation. Resting unstimulated B-cells in the presence of actinomycin D show a decrease in this ratio (Figure 6, left). This again confirms earlier findings of a preferential sensitivity of IgM synthesis to the inhibition of actinomycin D compared with the sum of all cellular protein syntheses (Melchers *et al.*, 1974; Andersson *et al.*, 1974) (Figure 6, left).

Addition of LPS to small, resting B-cells, preincubated for 20 min with actinomycin D, results in a stabilization of IgM synthesis. This is evident from a constant ratio of the rates of IgM synthesis over those of all cellular proteins over a subsequent period of 5 hr of stimulation, as compared to a decrease in this ratio in small B-cells exposed to actinomycin D, not stimulated by LPS (Figure 6, right). Stabilization of IgM synthesis in small lymphocytes after stimulation with LPS in the presence of actinomycin D became more pronounced when actinomycin D was added at the time of LPS addition. It was even more pronounced after 3 or after 7 hr of LPS stimulation (Figure 6, right). Control cultures not receiving LPS showed an unaltered sensitivity of their IgM synthesis to actinomycin D during the entire culture period.

Formation of Polyribosomes after LPS Stimulation in Small B-Lymphocytes. Small, resting B-lymphocytes from spleens of nude mice were cultured for 20 min in the presence or absence of actinomycin D. Thereafter, a mitogenic dose of LPS was added to one aliquot of untreated and actinomycin D treated cells, while the other aliquot of cells was kept without mitogen. After different periods of mitogenic stimulation, the cells were analyzed for their pattern of mono- and polyribosomes.

The results in Figures 7a and b show that LPS induces a redistribution of ribosomes from monoribosomes to polyribosomes. This redistribution occurs only with intact cells and cannot be induced by the addition of LPS to lysates of cells. The redistribution also occurs in the presence of actinomycin D, *i.e.* in the absence of RNA synthesis (Figure 7c). Mixing of lysates from LPS-stimulated and unstimulated cells yields the ribosome patterns expected from simple mixing without conversion of monoribosomes from the lysate of the unstimulated cells to polyribosomes and without breakdown of polyribosomes from the lysate of the LPS-stimulated cells to monoribosomes.

The change from monoribosomes to polyribosomes appears to be complete in 2–3 hr (Table II). The changes after mitogenic stimulation appear more pronounced in circulating small lymphocytes from the ductus thoracicus of nude mice (Table II).

We conclude that with our method of analysis, small resting B-lymphocytes appear to contain a large proportion of their ribosomes as monoribosomes and subunits. LPS induces polyribosome formation in small lymphocytes within the first hour of stimulation. This redistribution of ribosomes to polyribosomes does not depend on "de novo" RNA synthesis.

Discussion

Changes in biochemical parameters connected with IgM synthesis occur when small, resting B-lymphocytes are stimulated by mitogens (Melchers *et al.*, 1974; Melchers and Andersson, 1974a; see also introductory statement). Synthetic rate, turnover, size, carbohydrate composition, and

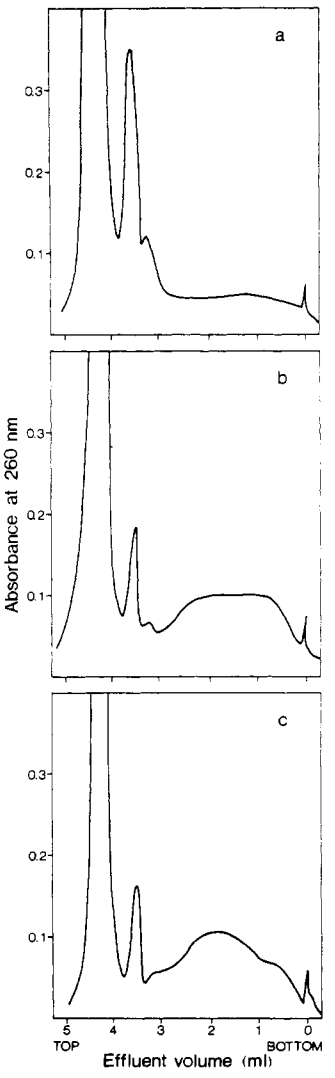

FIGURE 7: Polyribosome formation after addition of LPS to small, resting splenic lymphocytes from nude mice; 0.4 ml of lysate of 2×10^7: (a) resting small cells before addition of LPS; (b) resting small cells after addition of LPS (50 μg/ml, 5×10^6 cells/ml, 60 min at 37°); (c) resting small cells preincubated for 20 min with 5 μg/ml of actinomycin D, LPS added as above; analyzed on sucrose gradients as described in the Materials and Methods section.

TABLE II: Change of Distribution of Ribosomes from Monoribosomes to Polyribosomes after Mitogenic Stimulation of Small Lymphocytes either from Spleen or from Ductus Thoracicus with Time.

Cell Source and Time Period of LPS[c] Stimulation	Absorption at 260 nm[a] in Monoribosomes[b]	Polyribosomes[b]
Spleen, before LPS	0.25	0.15
Spleen, 20 min	0.15	0.15
Spleen, 60 min	0.1	0.3
Spleen, 120 min	0.09	0.3
Spleen, 180 min	0.12	0.29
Spleen + actinomycin D[d] before LPS	0.25	0.10
Spleen + actinomycin D,[d] 60 min	0.08	0.25
Ductus thoracicus before LPS	0.25	0.05
Ductus thoracicus, 120 min	0.05	0.30

[a] Measured from profiles such as the ones shown in Figure 7 by planimetration (see footnote *b*). [b] See Figure 7, monoribosomes in effluent volume (3.4–3.9 ml); polyribosomes in effluent volume (0–3.4 ml). [c] 20 μg/ml. [d] 5 μg/ml.

actinomycin D sensitivity of synthesis of IgM all change and can be taken as signs for B-lymphocyte activation and differentiation. The present results indicate that some of these changes can be detected very early, *i.e.* within the first hours, after stimulation. These early changes are mitogen dose dependent. It is evident by comparison to earlier results describing the dose dependence for the induction of DNA synthesis and the development of plaque-forming (PFC) IgM secreting cells (Melchers and Andersson, 1973, 1974b; Andersson *et al.*, 1973) that for two mitogens (LPS, fetal calf serum) the dose optimum for the stimulation to early changes, as measured in this paper (fetal calf serum, 10%; LPS, ~1 μg/ml), does not coincide with the dose optimum for the induction of DNA synthesis and for plaque-forming cell development. The dose optimum for the induction of DNA synthesis and for PFC development is lower for fetal calf serum (1%) and higher for LPS (50–100 μg/ml). For the third mitogen, the purified protein derivative of tuberculin, the dose optimum for the induction to early changes coincides with the dose optimum for the induction of DNA synthesis and of plaque-forming cell development (~50 μg/ml). Thus, are these early changes a sign for B-lymphocyte activation leading to DNA synthesis, proliferation, and the development of plaque-forming cells?

The discrepancies in the dose optima outlined above could be due to the activation of different populations of cells from nude spleen by different dose optima. Hence, the cells stimulated by optimal doses of LPS and fetal calf serum to early changes in IgM synthesis may *not* be the same cells which develop through DNA synthesis and proliferation into differentiated, IgM-secreting plaque-forming cells. As long as we cannot measure changes with single B-lymphocytes we are unable to prove this or rule it out.

The discrepancies in the dose optima could also be the consequence of a dual capacity of the B-cell mitogens to stimulate the *same* lymphocytes on the one side to DNA synthesis and proliferation and on the other side to differentiation, maturation, and development of IgM-secreting PFC. In a mitogenic response, reactions leading to proliferation may balance with reactions leading to differentiation and maturation (Melchers and Andersson, 1974c). Dose optima for stimulation to DNA synthesis and proliferation could be different from dose optima for stimulation to differentiation and maturation. A number of observations speak for such a balance of reactions. We have found (Andersson *et al.*, 1974; Melchers and Andersson, 1974b,c) that maturation of small resting B-cells can occur in the absence of DNA synthesis and proliferation, when these small B-cells are inhibited by hydroxyurea or cytosine arabinoside before they are stimulated by LPS. It is also interesting to note that the dose optimum for active secretion of IgM in the absence of DNA synthesis at 24 hr of mitogenic stimulation occurs between 1 and 10 μg/ml of LPS (Melchers and Andersson, 1974b), lower than the dose optimum for the induction of DNA synthesis and for the development of plaque-forming cells in uninhibited cultures (Andersson *et*

al., 1973) and thus nearer to the low doses, which are optimal for the induction of early changes measured in this paper. In fact, induction to maturation and active IgM secretion may be antagonistic to induction to proliferation in a B-cell. We have often observed a depression of the mitogenic response, measured as DNA synthesis at 48 hr and as plaque-forming cells at 72 hr of stimulation, at LPS doses between 1 and 10^{-2} μg/ml, when comparing it to cultures not receiving mitogen at all. Also, the different capacities of the three mitogens (LPS, the purified protein derivative of tuberculin, and fetal calf serum) to stimulate early changes *relative* to their capacities to stimulate a clonal development of plaque-forming cells are indications that the three mitogens can either affect different cells (see before) or the two reactions leading to proliferation and to differentiation with different strength.

Early changes in IgM synthesis occur in the presence of actinomycin D, *i.e.* in the absence of RNA synthesis. Mitogenic stimulation of early changes in IgM synthesis thus can be viewed as changes in the control of the expression of the genes for IgM on posttranscriptional levels. Earlier results indicate (Möller, 1972) that IgM synthesis is unstable in the absence of RNA synthesis; thus components such as messenger RNA's (mRNAs) for IgM, involved in IgM synthesis, may turn over rapidly in small resting B-lymphocytes. Mitogens induce the formation of polyribosomes even in the presence of actinomycin D (Figure 7). Through this mitogen-induced attachment of ribosomes to preexisting mRNAs, such mRNA molecules may be protected from degradation. It follows that the half-lives of such mRNAs are increased; consequently, the rate of protein synthesis coded for by these protected mRNAs may be increased simply by an increased number of mRNAs being translated at any given time. Since the rate of IgM synthesis is *selectively* increased over the rates of synthesis of all other cellular proteins (Figure 6; see also Melchers, 1972), stabilization of mRNAs may be selective for those mRNAs coding for IgM. We would like to emphasize that we have not yet shown, in this paper, that the polyribosomes formed after mitogenic stimulation contain IgM-specific mRNAs.

It is evident from earlier results describing the amplification of IgM synthesis in a B-cell after mitogenic stimulation (Melchers and Andersson, 1974a) that stimulation of resting B-cells to plasma cells actively secreting IgM cannot be expansion of the expression of IgM by regulation on the level of translation only. We expect that mitogenic stimulation of B-cells also leads to an increase in the rate of transcription of mRNAs coding for IgM. Preliminary results in our laboratory indicate, however, that such increased rates of RNA synthesis are detected in small B-cells only 3–5 hr after initiation of stimulation, *i.e.* later than the time in which we observe the early changes in IgM synthesis reported in this paper.

Actinomycin D treatment, by itself, shows no increase in rate of synthesis or intracellular concentration of IgM in small, resting B-cells, an effect sometimes termed "superinduction" (Tomkins *et al.*, 1972).

It is tempting to speculate that the formation of IgM-mRNA-containing polyribosomes may actually be the nucleus of the formation of membrane-bound polyribosomes of the rough endoplasmic reticulum. Membranous structures of the rough endoplasmic reticulum have been shown to be involved in active secretion of IgM (Rifkind *et al.*, 1962; De Petris *et al.*, 1963), and the membrane-bound polyribosomes of it appear to be involved in synthesis of secretory Ig (Williamson and Askonas, 1966; Shapiro *et al.*, 1966; Cioli and Lennox, 1973). The IgM molecules synthesized at the increased rate early after stimulation show all the biochemical characteristics of secreted IgM, *i.e.*, turn over rapidly and are secreted as 19S pentamers. Future experimentation will need a better distinction between free and possible early percursor forms of membrane-bound polyribosomes in order to test the hypothesis that mitogenic stimulation leads to the formation of membrane-bound polyribosomes.

Finally, it seems worth comparing B-lymphocytes with other cellular systems which, upon stimulation, undergo proliferation and differentiation to cells actively secreting proteins with specialized functions. It is apparent that many changes observed in hormone stimulation of chicken oviduct (Means *et al.*, 1971) or of mammary gland cells (Topper and Vonderhaar, 1974), or in serum stimulation of liver cells (Kaminskas, 1972), are very similar in B-lymphocytes in their molecular mechanisms and in their timing after stimulation. The comparison, more fully documented elsewhere (Melchers and Andersson, 1974d), indicates that mitogen-sensitive B-cells capable of proliferation and differentiation within 2–3 days to IgM-secreting cells are similar to resting cells *after* the primary hormonal stimulation and *before* hormonal recall to active secretion.

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Selective Action of Erythromycin on Initiating Ribosomes†

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ABSTRACT: Polysomal ribosomes of *Escherichia coli,* engaged in chain elongation, are shown to have a much lower affinity than free ribosomes for erythromycin (Ery), and they are 1000-fold less sensitive to inhibition by the antibiotic. The difference depends on peptidyl-tRNA: "pseudopolysomes," containing mRNA but no nascent peptide, are highly sensitive, like free ribosomes. Ery blocks initiating ribosomes not in formation of the initiation complex but in some later reaction, which is neither recognition of the next aminoacyl-tRNA nor peptidyl transfer of fMet. It is not certain whether the blocked reaction is the first translocation or some step in a later round of translation. The blocked ribosomal complexes are evidently unstable, for in cells inhibited by Ery the polysomes turn over extensively without protein synthesis, and they incorporate methionine but not valine. This cyclic blockade of initiation sites can explain the dominance of sensitivity over resistance in Ery^S/Ery^R heterozygotes.

Erythromycin A (Ery), a macrolide antibiotic, blocks protein synthesis in cells and extracts of sensitive bacteria, and it has been found to bind to the 50S ribosomal subunit (Taubman *et al.,* 1966; Wilhelm and Corcoran, 1967; Mao, 1967; Mao and Putterman, 1969; Teraoka, 1970). However, despite extensive studies during the past few years the mechanism of the inhibition remains unclear.

We have approached this problem by using purified polysomes of *Escherichia coli,* which can carry out peptide chain elongation but not reinitiation (Tai *et al.,* 1973b). The results show that Ery, like several other antibiotics (Tai *et al.,* 1973a; Wallace *et al.,* 1973, 1974), specifically inhibits initiating ribosomes and not elongating ribosomes. Moreover, the ribosomes are blocked by Ery shortly after initiation, and the blocked complexes are unstable, as shown by the turnover of polysomal ribosomes in cells. This cyclic blockade of initiation sites can explain the dominance of sensitivity over resistance to Ery in heterozygotes, just as similar findings have explained the dominance of sensitivity to streptomycin (Wallace and Davis, 1973) or to spectinomycin (Wallace *et al.,* 1974).

Materials and Methods

Bacterial strains, growth conditions, the preparations used for protein synthesis (S30 extracts, supernatant factors, crude initiation factors (IF), NH_4Cl-washed ribosomes, and phage R17 RNA), and the conditions of synthesis have been described (Tai *et al.,* 1973b).

Preparation of IF-Free Polysomes. Purified polysomes were prepared by gel filtration on Sepharose 4B from lysates of *E. coli* strain MRE600 (endogenous polysomes), and also from an S30 extract of *E. coli* strain S26 incubated with phage R17 RNA (R17 polysomes). Only preparations essentially free of reinitiation (Tai *et al.,* 1973b) were used.

Binding of [^{14}C]*Erythromycin.* Reaction mixtures (in 0.2 ml) contained buffer A (10 mM Tris-HCl (pH 7.6), 50 mM NH_4Cl, 8 mM $Mg(OAc_2)$, 1 mM dithiothreitol, about 2 A_{260} units (46 pmol) of ribosomes, and 0.75 μM [^{14}C]-*N*-methylerythromycin (11.3 μCi/mg = 13.5 cpm/pmol). After incubation 3 ml of buffer A was added and samples

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[1] Abbreviations used are: Ery, erythromycin; IF, initiation factor(s).