Induction of Immunoglobulin M Synthesis and Secretion in Bone-Marrow-Derived Lymphocytes by Locally Concentrated Concanavalin A

(mitogens/differentiation of lymphocytes/lymphocyte stimulation/polymerization of IgM/carbohydrate attachment to IgM)

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ABSTRACT Stimulation of bone-marrow-derived lymphocytes by locally concentrated concanavalin A involves first an increased intracellular synthesis of total protein and of immunoglobulin M at 10–14 hr, followed by the initiation of secretion of protein and of IgM at 24–30 hr after stimulation. Differentiation into immunoglobulin-producing and -secreting cells after stimulation is manifested by (a) a rate of IgM synthesis that increases continuously over total protein synthesis, and (b) an increased rate of IgM secretion with concomitant decrease of secretion of other proteins. Within stimulated bone-marrow-derived cells, IgM molecules were found mainly in their 7S form, together with some light-μ-heavy-chain precursors. Small amounts of polymerized 19S IgM are found associated with the cells. Only fully assembled 19S IgM was found secreted into the extracellular medium. Polymerization into the pentamer therefore takes place shortly before, or simultaneously with, secretion. Intracellular 7S IgM contains only glucosamine and mannose residues, with traces of fucose and galactose residues. Secreted 19S IgM has the full complement of carbohydrates with glucosamine, mannose, fucose, galactose, and N-glycolylneuraminic acid residues. During polymerization and secretion from the cells, IgM molecules therefore acquire all of the semiterminal and terminal residues of their carbohydrate groups.

Thymus-derived (T-) and bone-marrow-derived (B-) lymphoid cells cooperate in the immune response against most, but not all, antigens (1). The role of B-cells in the immune responses is reasonably clear: B-cells contain receptor immunoglobulins, predominantly of μ-heavy-chain class, in their outer membrane (2). Antigen, by attaching to receptor immunoglobulin, stimulates B-cells to proliferate and differentiate into immunoglobulin-producing and- secreting plasma cells (3). One cell produces only one structure of immunoglobulin (4); it may however switch during proliferation and differentiation from IgM- to IgG-production (5, 6). The role of T-cells is less clear: T cells show specificity for antigen (7), however, they contain very little, if any, receptor immunoglobulin on their outer membrane. T-cells may amplify or decrease the response to most antigens and thus serve a regulatory function in the expression of immune responses. After stimulation by antigen, T-cells proliferate but do not differentiate into immunoglobulin-forming cells. It is not clear whether the "helper" effect of T-cells is due to the production of B-cell-enhancing

factors (8), to the concentration of antigen and its presentation to B-cells (9, 10), or to their ability to induce the switch from IgM- to IgG-production in B-cells.

After antigenic stimulation of a normal lyphoid cell population containing T- and B-cells, B-cells change their regulatory state of transport and secretion of immunoglobulins. In antigen-sensitive small lymphocytes, immunoglobulin is synthesized and then deposited in the outer membrane, while in differentiated plasma cells, immunoglobulin is synthesized, transported, and then secreted from the cells.

Mitogens can mimic the action of antigen on lymphoid cells. Locally concentrated phytohemagglutinin (11) and locally concentrated concanavalin A (c-ConA) (12) stimulate B-cells to synthesize DNA, to proliferate, and to differentiate. While an antigenic determinant stimulates only a very small proportion of all lymphocytes (at best 0.02%) (13), mitogens stimulate between 20 and 70% of them. Mitogen-stimulated cells have been used, since biochemical studies of the cellular and molecular events connected with lymphocyte stimulation appear feasible only with a cell population the majority of which is able to respond to the stimulatory signal. It has been shown that pokeweed mitogen stimulates B-cells to produce and secrete IgM (14). In our studies, c-ConA has been used to stimulate B-cells from "nude" mice. These mice, as a consequence of a genetic defect, lack T-cells (15). Induction of an increased rate of IgM synthesis and the initiation of secretion of IgM after stimulation with antigen is measured to monitor the state of differentiation of B-cells in the absence of T-cells.

MATERIALS AND METHODS

Cell Sources. Spleen cells from mice with congenital thymic aplasia ("nude" mice) were used as a source of B-cells. Some of the mice were kindly supplied by Dr. I. Lefkovitz, Basel Institute for Immunology, Basel, Switzerland, and used at 4–8 weeks of age, while others were purchased from Bomholtgaard Ltd., Ry, Denmark. Single-cell suspensions were prepared as described earlier (16), and 1-ml cultures containing 5×10^6 living lymphocytes per ml of medium were set up in tissue-culture plastic petri dishes (no. 3005, Falcon Plastics, Los Angeles, USA) under described conditions (17).

Mitogen. 10 mg of concanavalin A (Miles Yeda Ltd., Rehovot, Israel), purified according to the procedure of Agraval and Goldstein (18), were dissolved in 1 ml of 0.1 M carbonate buffer (pH 8.5), containing 10 mol excess of α-methyl-D-mannoside (Calbiochem, San Diego, California) and mixed with 1 g of cyanogen bromide (Fluka, Buochs, SG, Switzerland)-activated Sepharose beads (Sepharose 4B, Pharmacia, Fine Chemicals, Uppsala, Sweden) (19). The Con

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Abbreviations: B-cells, bone-marrow-derived lymphocytes; T-cells, thymus-derived lymphocytes; c-ConA, locally concentrated Concanavalin A.

Nomenclature and abbreviations recommended by the World Health Organization [Bull. WHO. 30 (1964) 447] are used.

A–Sepharose beads were subsequently treated as described by Greaves and Bauminger (11). B-cells were activated with 1/100–1/1000 dilutions of the packed c-ConA bead material. Before incubation with radioactive leucine or sugars, cultures were harvested and cells were washed twice with phosphate-buffered saline (0.14 M NaCl–0.01 M potassium phosphate, pH 7).

Determination of DNA Synthesis. Cells were incubated with 2 μCi/ml of [^{3}H]thymidine ([*methyl-^{3}H*]thymidine, 5 Ci/mmol, The Radiochemical Centre, Amersham, U.K.), and radioactivity incorporated into trichloroacetic acid-precipitable material was determined as described (16).

Local Hemolysis in Gel Assay. Plaque-forming cells (PFC) were assayed by the method of Jerne *et al.* (20), as modified by Mishell and Dutton (21). The mitogen-induced PFC response against the 2,4,6-trinitrophenyl (Tnp) haptenic determinant was assayed with horse·erythrocytes treated with 2,4,6-trinitrobenzene sulphonate (22).

Cultures were harvested; cells from three dishes were pooled, washed, and suspended in balanced salt solution (21). Duplicate slides were made for each cell suspension, and direct PFC were developed by incubation for 3 hr at 37° with guinea-pig complement diluted 1/20 with balanced salt solution.

Determination of Immunoglobulin Synthesis. Cell suspensions at 5 × 10⁶ cells per ml of Eagle's medium without leucine were incubated with 60 μCi/ml of L-[*4,5-^{3}H*]leucine, (The Radiochemical Centre, Amersham, U.K. batch 26, 23 Ci/mmol), as described for myeloma plasma cells (23) with the following alterations: the medium did not contain any unlabeled leucine, but was supplied with 250 μM each of D-glucosamine, D-mannose, D-galactose, and L-fucose (Schwarz–Mann, Orangeburg, N.Y.) (24).

Separation of the labeled cells from their supernatant medium and lysis of cells with Nonidet P-40 (NP-40) (Shell Chem. Co., London, S.E.1.) nonionic detergent was done as described (23).

Cell lysates and medium supernatants were dialyzed extensively against phosphate–buffered saline and spun for 90 min at 20,000 rpm (100,000 × g) in a Sorvall RC-2B centrifuge. Radioactivity incorporated into total protein was measured in an aliquot of the samples directly, since it was found that trichloroacetic acid (final concentration 5% w/v) precipitated all the radioactivity in the samples.

Radioactivity incorporated into immunoglobulin was determined by precipitation with antisera. In principle, soluble complexes of radioactive mouse immunoglobulin and rabbit (anti-mouse immunoglobulin) antibodies are precipitated by pig (anti-rabbit immunoglobulin) antibodies (Sandwich technique). (We thank Dr. F. Franek, Czechoslovak Academy of Sciences, Prague, Czechoslovakia, for the supply of the pig serum.) Details of the precipitation procedures have been published (23). The following antisera, raised in rabbits by repeated injections of 1-mg amounts of protein in Freund's incomplete adjuvants (Difco) were used: (a) Antiserum against purified (25) 19S IgM (λ, μ) secreted by the plasma-cell tumor MOPC 104 E, was absorbed with IgG₁ (K, γ_1), purified from the serum of mice bearing the plasma-cell tumor MOPC 21 (26), with IgA (?, α), purified from the serum of mice bearing tumor S117 (27), and with K-type Bence–Jones light chain, purified from the urine of mice bearing MOPC-46
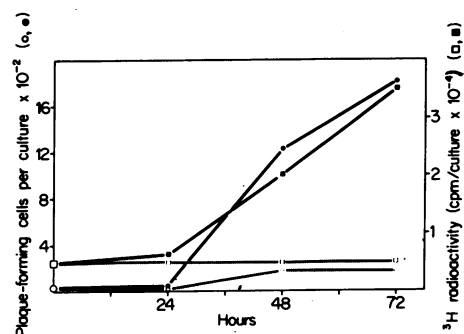

FIG. 1. Rate of [^{3}H]thymidine uptake (■, □) and appearance of antibody-forming cells (●, ○) in B-spleen lymphocytes in the presence (●, ■) or absence (○, □) of locally concentrated ConA (c-ConA).

tumor (28). Plasma-cell tumors MOPC 104E, MOPC 21, and MOPC 46 were kindly provided by Dr. M. Potter, National Cancer Institute, National Institutes of Health, Bethesda, Md.; plasma-cell tumor S117 was kindly provided by Dr. M. Cohn, Salk Institute for Biological Studies, La Jolla, Calif. (b) Antiserum against purified MOPC 21 IgG₁ (k, γ_1), was absorbed with MOPC 104 E 19S IgM (λ, μ), S117 polymeric IgA (?, α), and MOPC 46 K-type light chain. (c) Antiserum against β-galactosidase purified from *Escherichia coli* (29) was absorbed with MOPC 104 E 19S IgM, S117 polymeric IgA, MOPC 21 IgG, and MOPC 46 K-type light chain. Absorptions were all done with Sepharose 4B–immunosorbents (30) of the myeloma protein. Antiserum (a) was used to detect μ-specific determinants, antiserum (b) γ-specific determinants, and antiserum (c) was used to detect any unspecific precipitation. Data given in the results are corrected by subtraction of radioactivity precipitated by antiserum (c) from that precipitated by either (a) or (b). Radioactivity precipitated by antiserum (c) did not exceed 20% of the radioactivity precipitated by (a) and was as low as 5% for secreted protein from cells stimulated for 74 hr with c-ConA.

Polyacrylamide gels containing 0.1% sodium dodecyl sulfate, 0.5 M urea, and 0.01 M sodium phosphate (pH 7.1) were run at 6 mA per gel for 4 hr (31).

RESULTS

Proliferation and Synthesis of Antibody in B-Cells Stimulated by c-ConA. The time-course of the B-cell response to c-ConA is depicted in Fig. 1, where also the capacity of c-ConA to induce synthesis and secretion of immunoglobulins with demonstrable antibody activity against the 2,4,6-trinitrophenyl determinant (Tnp) is indicated. There is an increased rate of [^{3}H]thymidine uptake in c-ConA-stimulated cultures after 24 hr that is linear up to at least 72 hr of culture. Concomitant with this increase in proliferation, an increase in the appearance of antibody-forming cells (PFC) to Tnp is recorded.

Rate of Protein Synthesis and Secretion in Unstimulated and c-ConA-Stimulated B-Cells. B-cells from spleens of "nude" mice were incubated (a) in petri dishes to which c-ConA was added and (b) in petri dishes not containing the mitogen. After various periods of incubation the medium in the dishes was changed to medium containing radioactive leucine. Then the cells were incubated for another 4 hr. Rates of protein synthesis and secretion were measured as the amount of radio-
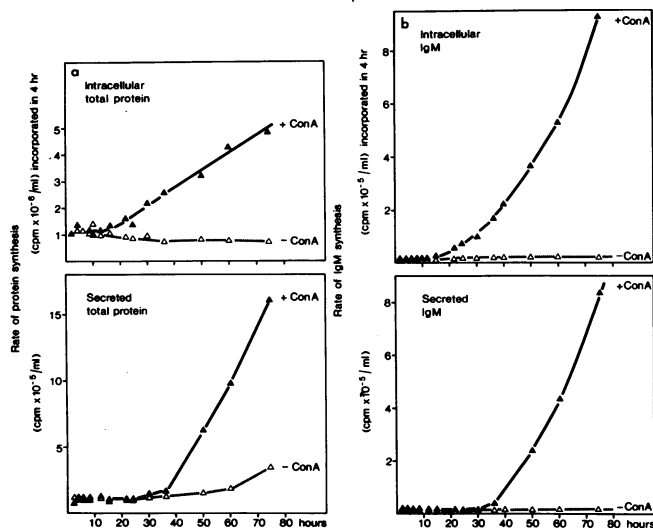
FIG. 2. Rate of total protein (a) and of IgM (b) intracellular synthesis and extracellular secretion in B-cells from "nude," athymic mice after various time periods of incubation in the presence (———) or absence (– – –) of c-ConA.

activity incorporated in 4 hr into nondialyzable, acid-precipitable material (see *Methods*). Incorporation of radioactive leucine by c-ConA-stimulated cells into protein was exponential during these 4 hr, at 24 hr after stimulation, whereas at 72 hr after stimulation incorporation was almost linear.

This result may indicate a decreasing rate of cell division between 24 and 75 hr after stimulation. Rates expressed in Fig. 2a for total protein and in Fig. 2b for IgM synthesis and secretion are not corrected for possible changes in the rate of cell division.

Rates of synthesis and secretion of total leucine-labeled protein by B-cells in the presence or absence of c-ConA are shown in Fig. 2a. In the absence of c-ConA, rates of synthesis and secretion stay practically unchanged. Protein secreted in 4 hr by unstimulated cells amounts to less than 10% of all protein synthesized during that time. In the presence of c-ConA, an increase in the rate of intracellular protein synthesis is observed. From an extrapolation of the data in Fig. 2a, we conclude that the rate begins to increase 10–14 hr after stimulation. Secretion of proteins is detectable between 24 and 30 hr after stimulation.

Rate of IgM Synthesis and Secretion. Rates of IgM synthesis and secretion were measured as the radioactivity incorporated from leucine in 4 hr into material precipitable by IgM-specific antiserum. Data shown in Fig. 2b are corrected for the amounts of radioactivity precipitated by a nonspecific antiserum (see *Methods*). An IgG-specific antiserum, absorbed with IgM, IgA, and light chains, did not precipitate any radioactivity significantly above that of the background precipitated with the nonspecific antiserum. Therefore, c-ConA stimulates only IgM, but not IgG, synthesis in this system.

Rates of synthesis and secretion of leucine-labeled IgM in the presence or absence of c-ConA are shown in Fig. 2b. In the absence of c-ConA the rate of IgM synthesis stays unchanged over 74 hr. Extracellular IgM at best amounts to 5% of the intracellular IgM in unstimulated cells. In the presence of c-ConA the rate of intracellular IgM synthesis increases, just as

does the rate of total protein synthesis, around 10–14 hr after stimulation. An increased secretion of IgM is detected between 24 and 30 hr after stimulation. From the results shown in Fig. 2a and b, we conclude that synthesis and secretion of IgM and of total proteins start to increase at the same time.

Data shown in Fig. 2a and b can be presented as ratios of the rates of synthesis and secretion of IgM to those of total protein (Fig. 3). The ratio of IgM synthesis to total protein synthesis increases from 0.015 at 10 hr to 0.17 at 74 hr after stimulation. The ratio of IgM secretion to secretion of total protein increases from 0.1 at 20 hr to 0.5 at 74 hr after stimulation. It shows that proteins other than IgM are secreted by B-cells early after stimulation.

Size of Intracellular and Secreted IgM in B-Cells Stimulated for 74 hr with Locally Concentrated ConA. The size of intracellular IgM and its precursor and of secreted IgM was determined by sedimentation analysis of the leucine-labeled material contained in the intracellular lysate and the extracellular medium of B-cells stimulated for 74 hr with c-ConA.

IgM and its precursors were identified in the fractions of sucrose density gradients by precipitation with specific antiserum (see *Methods*). Fig. 4 shows that secreted IgM is in the polymeric 19S form, with traces of light chains. Light chains were identified by their size in polyacrylamide gel electrophoreses. Intracellular IgM is predominantly in the 7S form, with some precursor material of lower S values at the top of the gradient, identified by polyacrylamide-gel electrophoreses as heavy–light-chain dimers and traces of free light chains. 10–15% of the intracellular IgM appears in the polymeric 19S form.

We conclude that IgM synthesized in c-ConA-stimulated B-cells accumulates as 7S subunits inside the cells. Polymerization of the subunits into pentamers occurs shortly before, or simultaneously with, secretion of the IgM molecules. Whether the small amount of 19S IgM found associated with the cells is truly intracellular in origin or only sticking to cells as already secreted molecules remains to be investigated.

Biosynthesis of the Carbohydrate Portions of IgM Synthesized and Secreted by c-ConA-Stimulated B-Cells. The time courses of synthesis and secretion of leucine-labeled and carbohydrate-labeled IgM in B-cells from "nude" mice stimulated for 72 hr with c-ConA (Fig. 5a–d) are strikingly similar to those observed with the mouse plasma-cell tumor MOPC 104 E
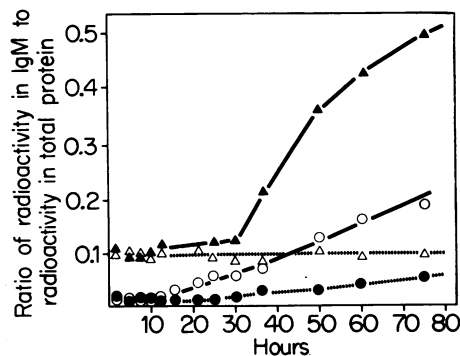

FIG. 3. Ratios of the rates of synthesis (O, ●) and secretion (△, ▲) of IgM to those of total protein synthesis and secretion (calculated from the data presented in Fig. 2a and b) at various times of incubation of B-cells in the presence (———) or absence (– – –) of c-ConA.

producing IgM (31, 32). Secretion of leucine-labeled IgM is somewhat faster than that observed with the tumor plasma cells; the curves for intracellular and extracellular IgM cross at 2 hr (Fig. 5a) rather than at 6 hr (32).

*Intra*cellular IgM, in the 7S precursor form, is only labeled by radioactive mannose (Fig. 5b). The label has been identified as mannose (85%) and glucosamine (10%) in IgM precipitated from lysates of B-cells labeled for 4 hr. *Extra*cellular, mannose-labeled (4 hr) 19S IgM contains radioactivity in mannose (40%), glucosamine (10%), galactose (15%), fucose (20%), and N-glycolyl-neuraminic acid (5%). Radioactive fucose and galactose do not label the intracellular IgM to any significant extent. However (Fig. 5c and d), extracellular IgM, all in 19S form, is labeled by these precursors. Radioactivity in IgM secreted after 4 hr of labeling with fucose and galactose was identified in the two experiments as fucose and galactose, respectively. No other sugar residues contained any appreciable radioactivity.

We draw the same conclusion for the structurally and functionally heterogeneous population of IgM molecules synthesized and secreted after c-ConA stimulation as we did for one homogeneous myeloma IgM (25, 31): the semiterminal and terminal carbohydrate residues of IgM, (galactose, fucose, and N-glycolyl-neuraminic acid) are acquired shortly before, or at the time, IgM is secreted from the cells and is polymerized into the 19S pentameric form. Our results make it likely that polymerization into 19S molecules and concomitant addition of semiterminal (galactose) and terminal (fucose, N-glycolyl-neuraminic acid) carbohydrate residues to IgM may be the rule for all IgM molecules secreted by stimulated B-cells.

DISCUSSION

Selective stimulation of IgM, but not of IgG, synthesis in mouse B-lymphocytes by pokeweed mitogen has been observed by Parkhouse, Janossy, and Greaves (14). c-ConA and pokeweed mitogen, therefore, have very similar effects on B-cells, which result in an increased synthesis and secretion of IgM. After 3 days of stimulation, the B-cell cultures produce and secrete as much IgM as does an IgM-producing and secreting mouse plasma-cell tumor (31, 33). This makes it likely that a large proportion of the cells in the stimulated cultures synthesize IgM. The average secretory capacity of a B-cell stimulated in our cultures for 74 hr can be calculated as the number of molecules of 19S IgM secreted per unit of time.
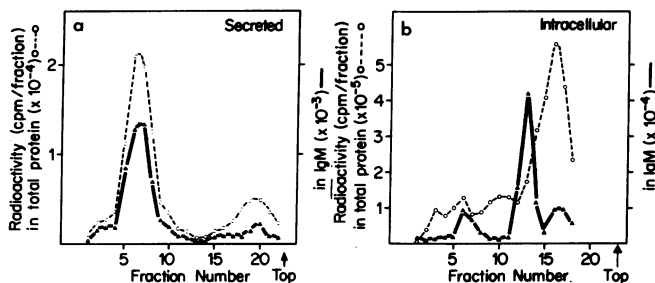

FIG. 4. Sedimentation analysis on 5–25% convex exponential sucrose gradients (ref. 25; sucrose density grade, RNase-free, Schwarz–Mann, Orangeburg, N.J.) of secreted (a) and intracellular (b) total protein (O- - -O) and of protein precipitable by μ-specific antiserum (▲———▲). All material precipitable by μ-specific antiserum in the gradient fractions contained heavy (μ) and light chains, as analyzed on polyacrylamide gels after full reduction and alkylation of the serologically precipitated material.
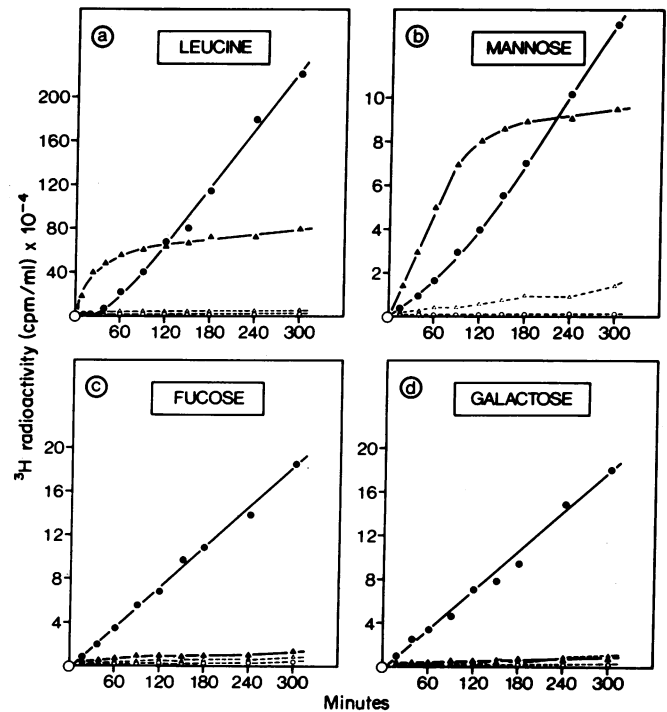

FIG. 5. Time-course of incorporation of (a) L-[4,5-³H] leucine (23 Ci/mmol; 60 μCi/ml) (b) D-[1-³H]mannose (100 μCi/ ml; 3.8 Ci/mmol, batch 6) (c) L-[1-³H]fucose (100 μCi/ml; 1.8 Ci/mmol, batch 4) (d) D-[1-³H]galactose (100 μCi/ml; 5.7 Ci/ mmol; batch 13); into intracellular (△, ▲) and secreted (O, ●) material serologically precipitable by μ-specific antiserum produced by B-cells from "nude", athymic mice in the absence (– – –) or presence (———) of c-ConA. Incubations b, c, and d were done in Eagle's medium without glucose (23), but in the presence of 250 μM each of glucosamine and fucose for the incorporation of radioactive galactose (d) and in the presence of 250 μM each of glucosamine and galactose (all from Schwarz–Mann, Orangeburg, N.J.) for the incorporation of radioactive fucose (c) (24). No additions were made to the glucose-free Eagle's medium for incorporation of mannose (b).

If we take the radioactivity in 19S IgM secreted at steady state (at 4 hr in Fig. 5a) and assume that the specific radioactivity of the estimated 600 leucine residues in the 19S IgM (molecular weight 900,000) is the same as that used in the incubation medium (23 Ci/mmol) (23), the average B-cell stimulated for 74 hr with c-ConA secretes 3.6×10^4 molecules per hr under our conditions. These values varied from experiment to experiment maximally by a factor of 5.

ConA forms complexes with certain polysaccharides, glycolipids, and glycoproteins (34, 35). The action of ConA on B-lymphocytes could be specific through the binding to IgM-receptor molecules on the surface (plasma) membrane of the cells. In fact, binding of ConA to the glycoprotein IgM in solution is best among the binding reactions with different classes of immunoglobulins (35), as measured by a precipitin reaction between ConA and immunoglobulins.

There are, however, many other glycoproteins from plasma membranes of lymphocytes that complex with ConA (36), so the action of ConA on B-lymphocytes can also be expected to be nonspecific through different membrane components acting as receptors. In both cases, however, a predominant number of the lymphocytes should be activated. Consequently, IgM molecules produced and secreted are

expected to be heterogeneous in structure, and are thus specific towards different antigens (37).

IgM synthesis and secretion increases over total protein synthesis with increasing time of stimulation. The ratio of the rates of synthesis and secretion may be taken as a parameter of the degree of differentiation into IgM-secreting plasma cells. In mature plasma cells this ratio should approach 1, i.e., IgM then remains the only protein to be secreted. For mouse myeloma plasma cells of the MOPC 104 E plasma-cell tumor, as an example, this ratio is 0.85 (Melchers, F., unpublished observations). If stimulation of B-cells by c-ConA is unspecific, as discussed above, it follows that small B-lymphocytes are genetically preprogrammed to undergo differentiation into IgM-producing and -secreting cells after either specific (antigen) or unspecific (mitogen) stimulation (see Fig. 3). One B-cell may be able to switch from IgM to IgG production (5, 6). B-cells from "nude" mice stimulated under our conditions with c-ConA are, however, not preprogrammed to switch, since only IgM is produced and secreted. Such a system could prove useful to study the factors that may influence the switch in immunoglobulin production.

It is remarkable that such a good agreement exists between the time course of the *in vitro* induction of DNA synthesis and the appearance of plaque-forming cells by specific antigen (17) and by unspecific mitogens (37). In accordance with these findings, secretion of IgM begins (24–30 hr after stimulation) when a rise in the number of plaque-forming cells is observed. It may well be that antigens activate lymphoid cells to undergo the same sequence of events observed in the activation of protein synthesis and secretion by mitogens, e.g., induction of intracellular synthesis at 10–14 hr, followed by secretion at 24–30 hr after stimulation.

Extensive morphological changes occur in the lymphocyte plasma membrane after stimulation, resulting in spot formation and capping of antibody-receptor molecules (38, 39). Such changes, occurring within a few hours after stimulation, are not accompanied by such extensive changes in protein synthesis that they could be detected by our method. We estimate that our method would identify radioactive IgM above the background of all other proteins made in B-cells in amounts as low as 1/20 of that secreted at 74 hr (Fig. 5a; 3.6 × 10⁴ molecules per hr per cell, see above), i.e., 2000 19S IgM or 10,000 7S IgM subunit molecules synthesized in 1 hr by one cell is the lower limit of detection in our experiments. If specific and unspecific stimulation of lymphocytes result in the same sequences of molecular events leading to proliferation and differentiation, increased *de novo* protein synthesis above this level of detection appears not to be a very early event after stimulation.

Note Added in Proof. We have found [Melchlers, F. & Andersson, J. (1973) *Transplant. Rev.* 14, in press] that bacterial lipopolysaccharide activates B-cells to increased IgM synthesis and secretion in a time sequence similar to that observed with c-ConA.

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