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Immunology

J chain synthesis and secretion of hexameric IgM is differentially regulated by lipopolysaccharide and interleukin 5

(B-cell differentiation/antibody secretion/IgM polymers)

TROY D. RANDALL*, R. M. E. PARKHOUSE†, AND RONALD B. CORLEY*‡§

*Department of Microbiology and Immunology, and ‡Comprehensive Cancer Center, Duke Medical Center, Durham, NC 27710; and †National Institute of Medical Research, Mill Hill, London, NW7 1AA United Kingdom

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ABSTRACT **Two functional polymeric forms of IgM can be produced by antibody-secreting B cells. Hexameric IgM lacks detectable J (joining) chain and activates complement 17-fold better than pentameric IgM, which usually contains one J chain per pentamer. Using the inducible B-cell lymphoma CH12, we determined if the synthesis of a particular polymeric form of IgM is a fixed property of B cells or can be altered. Lipopolysaccharide (LPS)-stimulated CH12 cells produced mixtures of IgM hexamers and pentamers, resulting in antibody with high complement-fixing activity. In contrast, interleukin-5-stimulated CH12 cells secreted predominately pentameric IgM, with a correspondingly lower lytic activity. Differences in lytic activity were due only to the amount of hexameric IgM in the secreted antibody. Interleukin 5 stimulated higher production of J chain RNA and protein than LPS, while LPS induced the highest levels of the secretory form of μ protein. The amount of hexameric IgM secreted was therefore inversely proportional to the level of intracellular J chain protein in the responding B cells. We conclude that the biologic function of IgM produced by B cells differs depending on how they are stimulated and that this difference may be regulated by the relative availabilities of J chain and secretory μ proteins during IgM polymerization.**

Classically, the IgM polymer is visualized as a pentamer of five H_2L_2 (H, heavy chain; L, light chain) monomeric subunits covalently associated by disulfide bonds and containing a single J or joining chain (1). J chain is a 15-kDa protein that is disulfide bonded to at least two of the μ H chains in the final polymer via the terminal cysteine, Cys-575, and/or Cys-414 (2–4). J chain does not contribute to the antigen specificity of the molecule, but it was originally hypothesized to be required for the polymerization and secretion of pentameric IgM (5–7). However, not all secreted IgM contains J chain or is pentameric. A hexameric form of IgM was identified in some of the earlier electron microscopic studies of polymeric IgM secreted by the plasmacytoma MOPC 104E, but the significance and function of this molecule was not investigated (8). Recently, we and others have shown that this hexameric form of IgM can account for a significant fraction of the secreted IgM from some B-cell lines and, like the pentamer, constitutes a functional molecule (3, 9). Unlike the pentamer, the hexamer is never observed with J chain. However, even pentamer assembly and secretion does not require J chain. A glioma cell line expressing transfected μ and κ molecules in the absence of J chain secreted IgM that consisted of both hexamers and pentamers (10). Furthermore, myeloma cells that expressed J chain as well as a transfected μ chain altered at either Cys-575 or Cys-414 secreted polymerized IgM that did not contain J chain (3, 4). These IgM polymers also consisted of mixtures of hexamers and pentamers. Davis *et al.* (3) demonstrated that the hexameric form of the IgM molecule was functional. They found that hexameric IgM activated the complement cascade in hemolytic assays up to 20-fold more efficiently than pentameric IgM, an observation confirmed and extended in our own studies (9). We found that inducible B-cell lines could secrete IgM hexamers, but the relative levels of hexameric to pentameric IgM varied depending on the cell line, thus leading to differences in the activity of the secreted IgM. Taken together, these studies show that J chain is not required for either assembly or secretion of IgM. It is unclear, however, if the production of hexamers is an intrinsic property of particular cell lines, such that they cannot polymerize IgM "correctly," or if the ratio of IgM hexamers and pentamers reflects a limited abundance or inaccessibility of J chain to the polymerizing IgM. When J chain is absent or in limited supply, a sixth IgM monomer might be inserted into the assembling polymer in place of J chain.

In the current study, we have tested this hypothesis. From earlier work using the inducible B-cell lymphoma CH12, we had found that synthesis of J chain RNA could be stimulated to a greater extent by lymphokine-containing supernatants than by lipopolysaccharide (LPS) (11). Here we demonstrate that while both LPS and interleukin (IL)-5 stimulate the differentiation of CH12 cells, IL-5 stimulates the production of J chain protein to a higher level than LPS. This differential stimulation correlates with the production of fewer IgM hexamers in the IL-5-stimulated population, thus lowering the biological activity of the secreted IgM, as measured by activation of the complement cascade. The functional significance of this result for our understanding of the regulation of the primary immune response is discussed.

MATERIALS AND METHODS

Cell Culture and Plaque-Forming Cell (pfc) Assays. The B-cell lymphoma CH12 was passaged *in vivo* as ascites cells and cultured as described (12). All cells were cultured in the presence of 5 μM 2-mercaptoethanol. CH12 cells were stimulated with LPS (*Escherichia coli* 055:B5; Difco) at 50 μg/ml and/or IL-2 and IL-5 (Genzyme) at the indicated concentrations (units as defined by the supplier). In some experiments, goat anti-mouse μ chain antibody (5 μg/ml; Sigma) was added. Individual antibody-secreting cells were enumerated in a pfc assay using bromelain-treated sheep erythrocytes (BrSRBC) as indicator cells as described previously (12). Antibody secretory rates were determined by washing and reculturing cells in replicate samples of 2×10^5 cells from each group in 1 ml of Dulbecco's modified Eagle's medium/

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Abbreviations: H, heavy; L, light; J, joining; LPS, lipopolysaccharide; IL, interleukin; pfc, plaque-forming cell(s); μ_m and μ_s, membrane and secretory μ chain; BrSRBC, bromelain-treated sheep erythrocytes.

§To whom reprint requests should be addressed at: Division of Immunology, Box 3010, Duke Medical Center, Durham, NC 27710.

fetal bovine serum (DMEM/FBS). Supernatants were taken at 2, 4, and 6 hr and the concentrations of IgM were determined by ELISA.

Electrophoresis and Western Blotting. Polymeric IgM was examined by electrophoresis in nonreducing 4% polyacrylamide gels under nondenaturing conditions as previously described (9). For Western blotting, nondenaturing gels were reduced in transfer buffer [25 mM Tris/0.28 M glycine/20% (vol/vol) methyl alcohol, pH 8.8] containing 0.5% 2-mercaptoethanol for 15 min and transferred onto Nitroplus 2000 (Micron Separations, Westboro, MA). Membranes were blocked with blotto (150 mM NaCl/10 mM sodium phosphate, pH 7.4/0.05% NaN_3/0.05% Tween 20/5% powdered milk), and probed with ^{125}I-labeled goat anti-mouse IgM antibodies (Sigma). To analyze μ and J chain abundance in CH12, cells were solubilized in loading buffer [25 mM Tris·HCl, pH 6.8/4 M urea/10% (vol/vol) glycerol/1% SDS/0.01% bromophenyl blue/0.5% 2-mercaptoethanol] and resolved on an SDS/10.5% polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with a rabbit anti-mouse J chain antisera (13), followed with a ^{125}I-labeled goat anti-rabbit IgG (Sigma) as a developing reagent.

ELISA and Hemolysis Assays. Quantitative ELISA for determining IgM concentrations and quantitative hemolysis assays for determining hemolysis of BrSRBC were performed exactly as described (14).

Northern Blotting. Total cellular RNA was isolated and analyzed by Northern blotting as previously described (11, 12). Probes were isolated as inserts from cDNA clones and labeled by "random priming" according to the supplier's specifications (Pharmacia) as described by Feinberg and Vogelstein (15). Clones used were pμ107 for μ chain, pAJ5 for J chain, and pHF-1 for actin, which have been described (11). The clone pSC33, containing a 500-base-pair (bp) 3′ portion of the κ constant region, was used as a κ probe (16).

RESULTS

Immunoglobulin and J Chain Gene Expression in LPS- and Lymphokine-Stimulated CH12 Cells. Since IL-2 and IL-5 have been shown to stimulate J chain production in BCL1 cells (17, 18), we stimulated CH12 cells with various combinations of IL-2 and IL-5 and compared immunoglobulin and J chain transcript levels with those from CH12 cells stimulated with LPS (Fig. 1). RNA from unstimulated CH12 cells exhibited two μ transcripts, with the μ_m transcripts equal or greater in abundance than μ_s transcripts, consistent with a replicating B-cell phenotype (12). As expected, LPS stimulation leads to significant increases in μ_s, κ, and J chain transcripts. IL-5 also stimulated production of these transcripts in CH12 cells and, interestingly, while increases in μ and κ were slightly less than those stimulated by LPS, J chain transcript levels were clearly higher. IL-2 appeared to have little effect on RNA levels in CH12 cells, either alone or in combination with IL-5. Addition of anti-μ to lymphokine-containing cultures had no additional effect on mRNA levels (not shown) or on secretory rates (see Table 1).

We next examined μ and J chain protein levels in extracts of stimulated CH12 cells by Western blotting. As shown in Fig. 2, the expression of μ was most significantly increased by LPS stimulation and the increase was confined to the secretory form of μ protein. Although IL-5 stimulation also increased μ_s protein, these increases were not as great as with LPS. Furthermore, the increases in μ were not equally reflected in the levels of J chain. While LPS did stimulate increases in J chain protein compared with unstimulated cells, the levels of J chain protein were clearly higher in cells stimulated with IL-5.

Differences in IgM Polymer Secretion from LPS- and IL-5-Stimulated CH12 Cells. We next determined if the IgM secreted by LPS- and IL-5-stimulated CH12 cells differed in its content of hexameric IgM. Resolution of secreted polymers on nondenaturing gels revealed that LPS-stimulated CH12 cells produced large amounts of both IgM hexamers and IgM pentamers (Fig. 3). In contrast, supernatants from IL-5-stimulated CH12 cells contained predominantly pentameric IgM. Cells that were cultured with medium alone or with IL-2 alone did not secrete polymeric IgM to any appreciable extent (Fig. 3; see also Table 1). These results suggest that the high levels of J chain that were induced by IL-5 in CH12 cells correlated with the secretion of relatively more pentameric IgM.

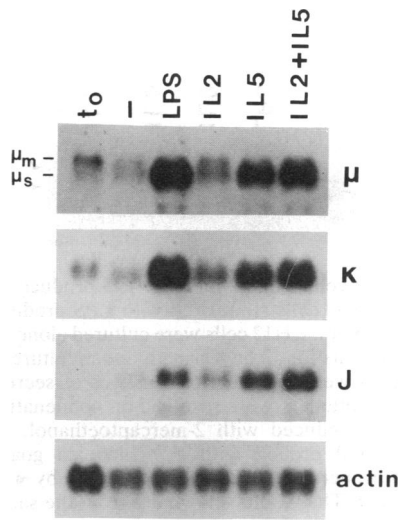

FIG. 1. Expression of immunoglobulin μ, κ, and J chain transcripts in LPS-, lymphokine-, or anti-μ-stimulated CH12 cells. CH12 cells were stimulated for 72 hr and the abundance of the designated transcripts was analyzed by Northern blot analysis. Total RNA (10 μg per lane) was extracted from CH12 cells that had been freshly isolated from ascites fluid (t_0), cultured in medium alone (−), or stimulated with LPS at 50 μg/ml, IL-2 at 50 units/ml, IL-5 at 25 units/ml, or both IL-2 and IL-5. Probes used are described in the text. The locations of the 2.7-kilobase (kb) membrane μ (μ_m) and 2.4-kb secretory μ (μ_s) transcripts are shown.

CH12 Cells Respond to IL-5 in a Dose-Dependent Fashion. To test if the secretory rates of IL-5-stimulated cells had effects on polymer formation, we performed a dose–response analysis of IL-5 stimulation of CH12 cells. As expected,

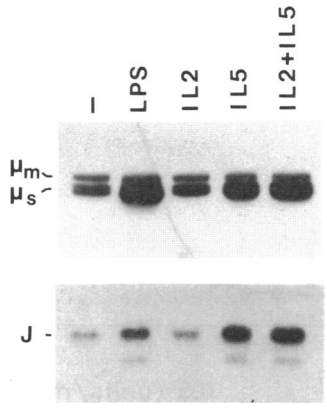

FIG. 2. Expression of μ_s and J chain protein after stimulation with LPS or with lymphokines. CH12 cells were cultured for 48 hr in the presence of medium alone (−), LPS, IL-2, IL-5, or IL-2 and IL-5 (concentrations as for Fig. 1). Extracts from 2.5×10^6 cells were prepared and resolved by electrophoresis in SDS/10.5% polyacrylamide gels. Proteins were transferred to nitrocellulose and probed either with a ^{125}I-labeled anti-μ antibody or with a rabbit anti-J chain antibody followed with a ^{125}I-labeled goat anti-rabbit IgG.

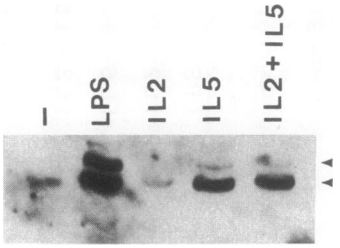

FIG. 3. CH12 cells stimulated with IL-5 produce predominately pentamers, whereas cells stimulated with LPS produce mixed hexamers and pentamers. CH12 cells were cultured alone (–) or with the appropriate stimulus for 48 hr, washed, and recultured for 6 hr, and the supernatants were collected for analysis of secreted IgM. The hexamers and pentamers were resolved on nondenaturing 4% polyacrylamide gels, reduced with 2-mercaptoethanol, transferred to nitrocellulose, and probed with a ^{125}I-labeled goat anti-μ. The locations of hexamers and pentamers are shown by ◄ (hexamers are the upper bands). The results shown are from the same experiment as in Fig. 2.

CH12 cells responded to IL-5 in a dose-dependent manner (Fig. 4). The secretory rates were increased above background at concentrations of IL-5 as low as 5 units/ml and leveled off at concentrations of IL-5 between 60 and 250 units/ml. At these concentrations, the secretory rates were only slightly lower than those of LPS-stimulated cells.

Fig. 5 shows the polymeric IgM content of supernatants from CH12 cells stimulated as indicated in Fig. 4. Cells that were incubated in medium alone secreted low amounts of IgM, and, as expected, LPS-stimulated cells produced both hexamers and pentamers, with the hexameric form constituting 30–40% of the total IgM secreted. CH12 cells stimulated with IL-5 responded by the secretion of predominately pentameric IgM at all doses tested, even at the higher concentrations of IL-5, which induced IgM secretory rates comparable to those induced by LPS. Interestingly, the ratio of IgM hexamers to pentamers was constant at all concentrations of IL-5 used and similar to the 5–10% observed as background in unstimulated CH12 cells.

Hemolytic Activity of Secreted IgM Is Changed by the Amount of Hexamers Secreted. IgM hexamers are 17 times more efficient at activating complement than are IgM pentamers in hemolysis assays (3, 9). Thus, secreted IgM from CH12 cells stimulated with LPS should be more efficient in its hemolytic activity than IgM secreted by IL-5-stimulated CH12 cells. Consistent with this, we observed that hemolytic plaques from LPS-stimulated CH12 cells were significantly larger than plaques produced by the same cells stimulated with IL-5 (data not shown). To eliminate the possibility that differences in plaque size were due to different secretory rates, we measured the secretory rate of cells from LPS-stimulated cultures and compared these with the secretory rates of cells stimulated with IL-5 at 25 units/ml (Table 1). Although the overall secretory rate was highest in cells from the LPS-stimulated cultures, more cells responded to LPS by differentiating into pfc. When secretory rates were expressed relative to the numbers of pfc in the cultures, no difference between the rates of cells stimulated by LPS or IL-5 was found. These rates were calculated to be in the range of 3000–4000 molecules per cell per sec and are in the same range as those reported for myeloma and hybridoma cells (19). It is interesting to note that the rates of secretion in all cultures were similar in magnitude, when calculated relative to the fraction of pfc in the population. This was true even in cultures in which cells were not stimulated, such as those that were incubated with anti-μ or IL-2 (see Table 1 and Fig. 6*B*). This suggests that individual cells differentiate in an "all-or-none" manner, irrespective of which stimulus causes differentiation.

As a direct demonstration of changes in hemolytic activity, we compared the hemolytic efficiency of the secreted IgM from different cultures. The quantity of IgM in the supernatants was determined by ELISA, and the ability of the IgM to lyse BrSRBC was determined by quantitative hemolysis

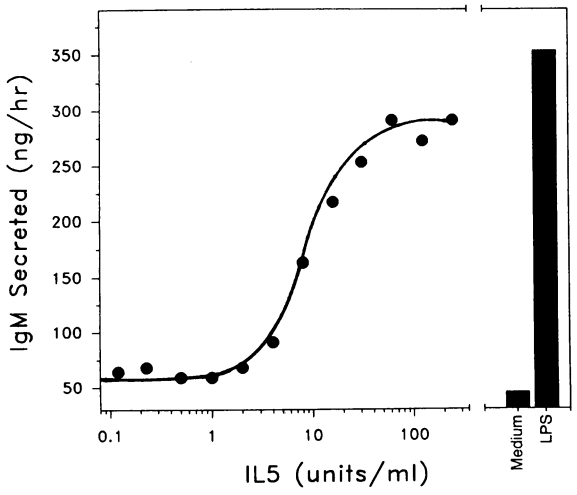

FIG. 4. CH12 cells respond to IL-5 in a dose-dependent fashion. CH12 cells were cultured with medium, LPS, or IL-5 at the designated concentrations. After 48 hr, the cells were washed and recultured for 6 hr in the absence of any lymphokine. IgM secreted into the supernatants was quantitated by ELISA and the rate of secretion (ng/hr) was calculated. In experiments not shown, the secretory rates of unstimulated and stimulated CH12 cells were determined to be linear over the 6-hr time course.

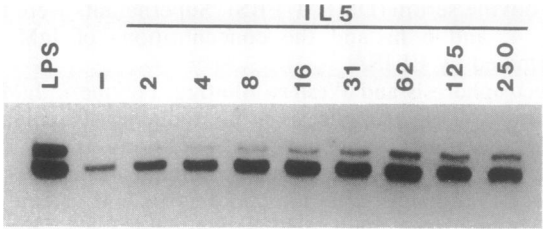

FIG. 5. IL-5-induced polymeric IgM from CH12 cells is predominately pentameric. Supernatants from CH12 cells cultured in the presence of LPS (lane 1), medium alone (lane 2), or IL-5 at the indicated concentrations (units/ml) were examined by nondenaturing PAGE. The proteins were reduced with 2-mercaptoethanol in the gel, transferred to nitrocellulose, and probed with ^{125}I-labeled anti-μ. Hexameric and pentameric IgM are noted by ◄.

Table 1. Secretory rates of stimulated CH12 cells

Culture	Antibody secretion		IgM secretion*	
	pfc, %†	ng/hr‡	per cell per sec	per pfc per sec
Medium alone	0.8	22	97	—§
LPS	53.1	365	1607	3026
IL-2	2.7	20	88	3259
IL-5	26.6	192	845	3177
IL-2 + IL-5	24.4	159	700	2869
α-μ	3.9	32	141	3615
α-μ + IL-2	2.6	17	75	2885
α-μ + IL-5	23.5	226	995	4234
α-μ + IL-2 + IL-5	21.0	159	700	3333

α-μ, Anti-μ.
*Calculated and expressed as secretion of IgM monomers, assuming a monomeric subunit size of 190 kDa.
†Expressed as percent of viable recovered cells.
‡IgM secreted by 2×10^5 cells.
§Insufficient pfc to calculate an accurate figure.

assays (14). The lytic efficiency was calculated as the ng of IgM required to give an A_{405} of 1, and the relative efficiency was established, with that of the IgM in the supernatants from LPS-stimulated cells taken as 1. Supernatants from IL-5-stimulated CH12 cells were in all cases less efficient at lysing BrSRBC than supernatants from LPS-stimulated cells (Fig. 6). In two different experiments, IgM from lymphokine-stimulated cells were only 25–45% as efficient as IgM from LPS-stimulated cultures. Typically, IgM from LPS-stimulated cells is 30–40% hexameric, while IgM from IL-5-stimulated cultures is 5–10% hexameric. Using these ratios and the calculated 17-fold difference in complement activation (9), the same quantity of IgM from LPS-stimulated cells would be predicted to be 2.5–4.0 times more active than IgM from IL-5-stimulated cells. This is in good agreement with the experimental results. Thus, the differences in plaque sizes and hemolytic activity of IgM from the different cultures is due only to the relative mix of hexameric and pentameric IgM secreted by the responding cells.

DISCUSSION

IgM has two functions that make it of particular importance in combating infectious diseases: its high capacity for activating the complement pathway and its ability to bind to the polymeric immunoglobulin receptor and be transcytosed into exocrine secretions (20, 21). Both of these properties depend on the ability of IgM to polymerize prior to secretion. Thus, the processes that control the polymerization of IgM play a critical role in the immune functions of the B cell. The IgM polymer is usually depicted as a pentamer of H_2L_2 subunits with one J chain subunit per pentamer (1). This historic view of IgM structure has been challenged by recent data showing that the incorporation of J chain into polymerizing IgM is not essential for IgM polymer secretion (3, 9, 10), and that hexameric IgM lacking in J chain possesses at least one of the

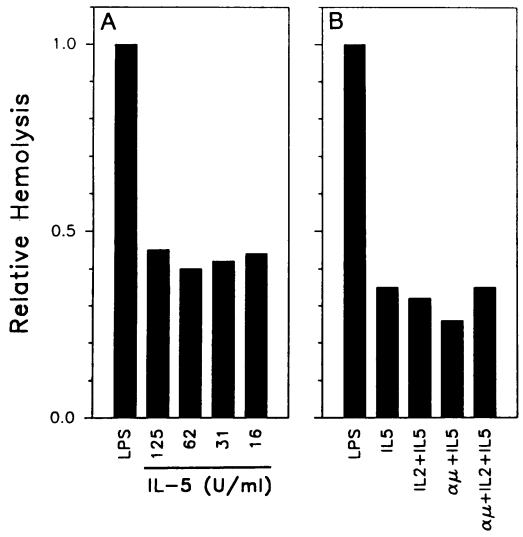

FIG. 6. Hemolytic activity of IgM secreted by LPS-stimulated CH12 cells is significantly greater than that of the IgM secreted by IL-5-stimulated CH12 cells. The hemolytic activities of supernatants from CH12 cells stimulated as shown (see Figs. 3 and 5) were determined by quantitative assays (14). The concentration of IgM in the supernatants was quantitated by ELISA, and the amount of IgM required to give an A_{405} of 1 (which is on the linear portion of the curve) in a hemolysis assay was calculated. The relative hemolytic activities were then determined, with the hemolytic activities of the IgM in the LPS-stimulated supernatants taken as 1.0. Results from two different experiments are shown. In experiment A, 34 ng of IgM from LPS-stimulated cells was required for hemolysis, while in experiment B, 58 ng was required. $\alpha\mu$, Anti-μ.

important functions of the IgM polymer, efficient activation of the complement cascade (3, 9).

The current work was carried out to determine if the mix of hexameric and pentameric forms of IgM secreted by B cells is an intrinsic phenotype of differentiating B cells or if the relative abundance of IgM components, such as μ_s or J chain, can alter this mix. We found that IL-5 stimulation of a clonal population of B cells, CH12, resulted in the predominant production of IgM pentamers. However, the stimulation of these same cells with LPS resulted in the production of a significant fraction of hexameric IgM, usually 30–40% of the total. This increased hexamer production of LPS-stimulated cells led to a 3- to 4-fold increase in the specific activity of the secreted antibody, consistent with the calculated predictions.

At the very least, these data demonstrate that the mix of IgM polymers capable of being secreted by an inducible B-cell lymphoma, CH12, is not a fixed property of the cells but can be influenced by external factors, including the mode of stimulation. How generally applicable are these results to normal B cells? At this time, we cannot directly address this question. Differences in glycosylation and charge affect the migration of IgM polymers in gels, and we have been unable to adequately resolve IgM hexamers from pentamers in normal serum. Nevertheless, several lines of evidence indicate that the ability to produce different polymeric forms of IgM is not limited to particular subsets of B cells. IgM hexamers are produced by various B cell lines, including MOPC 104E (8), BCL1, and WEHI 231 (unpublished data), and are produced by human B cells, including Epstein–Barr virus-transformed B-cell lines (22). Hexameric IgM has also been isolated from a patient with Waldenström macroglobulinemia (23), indicating that this form of IgM polymer can be found in the circulation. In our own studies, despite the inability to completely resolve hexamers and pentamers from normal mouse serum, we have observed mixtures of IgM hexamers and pentamers produced by B cells from mice expressing immunoglobulin μ and κ transgenes (unpublished).

What controls the final mix of IgM polymers secreted by B cells? CH12 cells respond to IL-5 by increasing J chain levels well above those seen in LPS-stimulated cells (Fig. 2). Furthermore, these cells secrete almost exclusively pentameric IgM (Fig. 3). In recent experiments, we have found that IL-6 also induces high levels of J chain and the predominant secretion of IgM pentamers (unpublished data). Since the secretory rates of LPS- and IL-5-stimulated cells are very similar, it is unlikely that the production of hexamers is simply a phenomenon of a high secretory rate "overloading" the secretory pathway. However, our data showing that high levels of J chain correlate with IgM pentamer formation suggest that J chain may influence the type of polymers that are formed. *In vitro* data on IgM assembly support this conclusion. Kownatski (24) found that purified IgM monomers could reassemble in the absence of J chain but consisted of large amounts of hexameric as well as pentameric IgM. The addition of purified J chain during reassembly led to the formation of essentially pure pentameric IgM, suggesting that the formation of pentamers containing J chain is preferred. We suggest a model in which the assembly of IgM monomers into an open pentameric IgM is a rapid process (Fig. 7). According to the data of Sitia *et al.* (25), this molecule would not be secreted, since the free cysteine residues would cause it to be retained. To be secreted, the free terminal cysteines in the IgM molecule must be used by the covalent association with J chain or with other μ chains. It is also likely that other factors, such as the abundance of disulfide isomerases (26, 27) or other proteins involved in the polymerization process, may influence the ratio of these two polymeric forms.

Why should the immune system have J chain at all, when it is not needed for the assembly or secretion of IgM, and IgM

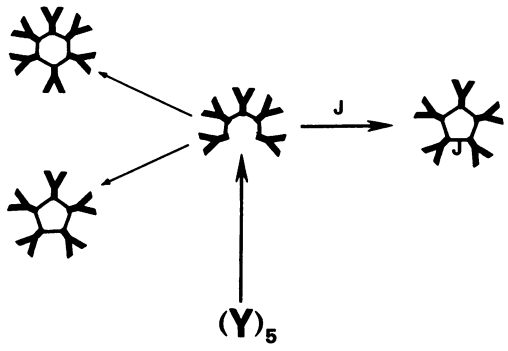

FIG. 7. Proposed scheme for IgM assembly into pentamers containing J chain and pentamers and hexamers not containing J chain. See *Discussion* for explanation.

pentamers with J chain are less potent at complement activation than hexameric IgM without J chain? There must be properties intrinsic to immunoglobulin polymers containing J chain (both IgM and IgA) that are absent from polymers that lack J chain. Eskeland, Brandtzaeg, and co-workers (23, 28, 29) have hypothesized that J chain mediates the interaction of polymeric IgA and IgM with the poly immunoglobulin receptor, thus allowing the transcytosis of these polymers across epithelial cells into secretory compartments (21, 30). In the case of IgM, the large benefit gained from the resulting secretory immunity may far outweigh the benefits of having increased lytic activity in the serum. It is also possible that IgM hexamers may be detrimental to the immune system under certain conditions. For example, if low-affinity "natural antibodies" become highly lytic, unwanted damage may occur. Finally, the synthesis of J chain by plasma cells not secreting polymeric immunoglobulin (13, 31) might suggest an as yet undiscovered role for J chain.

The ability to regulate J chain synthesis, and thus alter polymer production, may reflect an adaptive function of the immune system, which fine-tunes the relative abundance of the different polymeric forms of IgM to the type of pathogen involved in stimulation. In bacterial infections, the most effective response is clearance through complement activation, either by direct lysis or by opsonization. The increased production of IgM hexamers in immune responses to bacteria could therefore bestow a protective advantage. Interestingly, immune responses to many bacterial surface polysaccharides tend to be T-cell-independent and, like the response stimulated by LPS alone, might be expected to result in the increased production of hexameric IgM. On the other hand, the elimination of pathogens such as viruses do not normally require complement activation, since the binding of antibody alone usually leads to viral neutralization. As a result, production of hexamers would not necessarily be advantageous. Viral immune responses are usually helper T-cell-dependent, which should therefore promote increased J chain synthesis through the action of T-cell-derived lymphokines.

From these considerations, we suggest that there are three mechanisms whereby B cells can modify the biologic activity of their secreted antibody. The first two, isotype switching and somatic hypermutation, are predominately mechanisms that affect T-cell-dependent immune responses and are most evident in secondary B-cell responses (32–34). The third, which we propose here, is the alteration of IgM hexamer and IgM pentamer production, which may have evolved to influence the biologic activity of primary antibody responses, and may distinguish the activity of antibodies secreted in T-cell-dependent and -independent immune responses. *In vivo* analysis will be necessary to directly test the validity of this hypothesis.

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