

Conservation of structural and functional domains in complement component C3 of *Xenopus* and mammals

(evolution/sequence homology/major histocompatibility complex/properdin binding site/Arg-Gly-Asp sequence)

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ABSTRACT The cDNA sequence and the deduced amino acid sequence of the M_r 34,000 C-terminal fragment of *Xenopus laevis* complement component C3 are presented. The sequence of *Xenopus* C3 has 57% nucleotide identity to the corresponding sequence of human C3 and \approx 49% amino acid identity to C3 from human, mouse, and rabbit. The *Xenopus* C3 sequence shows clusters of high and of low similarity to the mammalian C3 sequences. One of these regions of high similarity represents the domain of mammalian C3b involved in the binding of properdin, a regulator of the alternative pathway of complement activation. It is not clear whether the other highly conserved regions are involved in binding to other C3 ligands. The *Xenopus* C3 sequence completely lacks the Arg-Gly-Asp sequence, which has been suggested to be the recognition site of the human complement receptor type 3 on the iC3b fragment of human C3. The *Xenopus* C3 gene is shown not to be linked to the *Xenopus* major histocompatibility complex, as is also the case in mammals. Since the gene of the related molecule C4 is MHC-linked in both mammals and *Xenopus*, the C3 and C4 genes may have separated before *Xenopus* and mammals speciated.

The third component of complement (C3) plays a central role in both the classical and alternative pathways of complement activation by interacting with numerous serum and surface complement proteins (1, 2). In addition to being important for complement activation, C3 has been found to play a significant role in inflammatory processes and in the immune response (1, 3).

Xenopus has both classical (4) and alternative (5, 6) pathways of complement activation. The complement proteins C3 (7) and C4 (4) isolated from *Xenopus* serum have subunit structures similar to mammalian C3 and C4. Human C3 fragment C3b is cleaved by *Xenopus* serum in a way similar to its cleavage by human serum, suggesting the presence of the regulatory proteins factors I and H in *Xenopus* serum (8). Receptors for *Xenopus* C3 fragments have been found on the surface of *Xenopus* macrophages (9), although it is not clear whether these are the *Xenopus* homologs of mammalian macrophage complement receptor type 1 (CR1) or 3 (CR3). Since the *Xenopus* C3 molecule shares many features with mammalian C3, including binding to mammalian CR1, properdin, and C5 (10), comparison of the *Xenopus* C3 sequence with mammalian C3 sequences may reveal regions that are involved in binding to its many ligands as well as the structural elements of these interactions. Such regions are expected to be relatively conserved, since their rate of evolutionary divergence would be constrained by the rate of evolution of their ligands.

This paper describes the isolation and characterization of a cDNA clone representing the carboxyl end of the α chain of the *Xenopus* C3 molecule.* The *Xenopus* C3 sequence shows \approx 49% amino acid identity to the corresponding sequence of mammalian C3 and has clusters of conserved sequences that probably represent regions important for the structure and function of the C3 molecule. In addition, the gene for *Xenopus* C3 is shown not to be linked to the *Xenopus* major histocompatibility complex (MHC), a result with implications for understanding the evolution of complement genes and the MHC.

MATERIALS AND METHODS

Library Construction. RNA was prepared from the liver of an adult female *Xenopus laevis* (11) and a cDNA library was constructed in the expression vector λ gt11 (12) essentially as described (13).

Preparation of Anti-*Xenopus* C3 Antibody. A rabbit anti-*Xenopus* C3 antiserum was prepared by using zymosan-C3b/iC3b particles as described (7). The anti-*Xenopus* C3 antibody was affinity-purified on zymosan coated with α -chain fragments of *Xenopus* C3. The coated zymosan was prepared by incubation in *Xenopus* serum and washing with 0.1% sodium dodecyl sulfate (SDS) containing 2% 2-mercaptoethanol.

Screening of Library. The cDNA expression library was screened essentially as described (14). The filters were incubated successively with biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories) and a complex of avidin and biotinylated horseradish peroxidase (Vector Laboratories) and were developed with the peroxidase substrate 3,3'-diaminobenzidine (Sigma).

Immunoblots. Immunoblots of normal *Xenopus laevis* serum were prepared as described (15). The blots were treated in the same way as the filters used in the library screening.

Sequence Determination. The *Eco*RI inserts were subcloned into the Bluescript plasmid vector (Stratagene) and several nested deletion constructs were prepared. Sequence was determined from plasmids (16) with the use of Sequenase (United States Biochemical) and was compiled with the MICROGENIE program (Beckman). Protein alignments were done with a program (written by C. Steinberg, Basel Institute for Immunology) using the Needleman-Wunsch algorithm (17).

Southern Blot. DNA was isolated from *Xenopus* erythrocytes and used as described (18). The probe was excised from the pC3-27 subclone of C3-27 by digestion with *Bam*HI,

Abbreviations: C $_n$, complement component n ; MHC, major histocompatibility complex; CR $_n$, complement receptor type n ; α_2 M, α_2 -macroglobulin; RGD, Arg-Gly-Asp.

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04493).

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purified by agarose gel electrophoresis (19), and labeled to a specific activity of 10^9 cpm/ μ g (20).

RESULTS

Isolation of a *Xenopus* C3 cDNA Clone. Activation of the alternative pathway of complement by zymosan leads to the covalent binding of C3b to zymosan through its reactive thioester site on the α chain (21). This property was used to purify *Xenopus* C3 fragments and to produce anti-*Xenopus* C3 antibodies. Electrophoresis of fragments eluted by boiling a zymosan-C3b/iC3b preparation in the presence of SDS and 2-mercaptoethanol showed the expected β chain (M_r 85,000) and the M_r 34,000 C-terminal fragment of the α chain of iC3b (Fig. 1A). Affinity purification was necessary to reduce the background (Fig. 1B, lane 1). The affinity-purified antibody reacts with the uncleaved α chain as well as α' chain and its M_r 34,000 C-terminal fragment (Fig. 1B, lane 2). We do not understand why this antibody did not also recognize the M_r 81,000 N-terminal α -chain fragments of iC3b (7).

Screening of the λ gt11 expression library with the affinity-purified antibody resulted in one positive phage clone, C3-27 (Fig. 1C). To verify that this clone actually represents *Xenopus* C3, it was plated at high density on several Petri dishes and the filters were used to affinity-purify the anti-*Xenopus* C3 antibody. This antibody recognized the same protein species as the antibody purified on zymosan-bound C3 α chain (Fig. 1B, lane 3). This showed that C3-27 is in fact a *Xenopus* C3 clone. It also placed this clone at the C terminus of the α chain, since the antibody selected on C3-27 recognized the M_r 34,000 C-terminal α -chain fragment of the *Xenopus* iC3b.

Sequence Analysis. To obtain longer cDNA C3 clones, a 425-base-pair *Bam*HI fragment was purified from the pC3-27 subclone of the *Eco*RI insert of C3-27. Approximately 200,000 clones were screened by hybridization, and of the resulting 16 positive clones, C3-39 was the longest (997 base pairs). The inserts in this library, as well as in four other

libraries tested, appear to be considerably shorter than the full-length C3 mRNA, which is expected to be about 5.5 kilobases (kb).

The full pC3-27 sequence was determined, as well as additional regions of pC3-39. When the assembled nucleic acid sequence of this region of *Xenopus* C3 is aligned with the human C3 sequence (Fig. 2), it shows 57% nucleotide identity.

In Fig. 3 the cloned region of *Xenopus* C3 is aligned with the corresponding regions of the human mRNA and human protein. The human mRNA is 5.5 kb and encodes a protein that is cleaved once to give the α and β chains of mature C3 (2). The *Xenopus* C3 sequence aligns at the 3' end of the mRNA and at the C terminus of the mature α chain.

In Fig. 4 the deduced *Xenopus* C3 amino acid sequence is aligned with the available mammalian sequences, as well as with the sequences of the structurally related molecules C4 (26), C5 (27), and α_2 -macroglobulin (α_2 M) (28). All sequences were aligned against the *Xenopus* sequence. The *Xenopus* C3 amino acid sequence is about equally similar to the three mammalian C3 sequences and significantly less similar to the C4, C5, and α_2 M sequences (Table 1). Amino acid residues at positions 48 (cysteine), 63 (aspartic acid), 69 (glycine), 80 (leucine), and others (in reversed print in Fig. 4) are conserved in all complement sequences aligned, although these proteins have diverged considerably in function. These residues are mostly either cysteine or hydrophobic and may be necessary for the overall structural integrity of this family of proteins. In addition to the cysteines found in the M_r 40,000 mammalian C3 fragment, *Xenopus* C3 has three additional cysteines at positions 25, 27, and 33. In mammalian C3 one of the cysteines is disulfide-bonded to the M_r 67,000 N-terminal α -chain fragment. The M_r 34,000 C-terminal fragment of the α -chain is smaller than the mammalian M_r 40,000 fragment, so *Xenopus* C3 may have a different site of factor I cleavage. A potential Arg/Ser cleavage site is located at residues 37 and 38, and a fragment of $M_r \approx 32,000$ would result from cleavage there. If this is the case, the additional cysteines would not reside in the M_r 34,000 C-terminal fragment.

Several structural features shared by these proteins are apparent from these comparisons. The properdin-binding region of human C3 (residues 84-116) (29), which has been further localized to 9 amino acids (residues 105-113; M. Daoudaki and J.L., unpublished observations), shows high degree of similarity to C3 of other species, and much less similarity to the non-properdin-binding proteins C4, C5, and α_2 M. Interestingly, the tripeptide RGD sequence in the human C3 sequence, reported to be the recognition site of CR3 on iC3b (30), is not completely conserved in the mouse and rabbit sequences and is absent in the *Xenopus* sequence. However, considerable conservation exists to the C-terminal side of the RGD site.

The *Xenopus* C3 Gene Is Not Linked to the MHC. A Southern blot was done with DNA from a family of interspecies hybrids from a cross of *X. laevis* and *X. gilli* (Fig. 5). These frogs make diploid eggs that develop upon stimulation with irradiated sperm, and are thus maintained as clones. The MHC types of these cloned frogs were determined by grafting and by mixed lymphocyte culture. In this family there are four MHC haplotypes, *a*, *b*, *c*, and *d*, which segregate independently (31). The DNAs were digested with *Eco*RV and *Pst* I, and the blot was probed with a fragment comprising 425 base pairs 5' of the *Bam*HI site from the pC3-27 subclone (see Fig. 3). The LG6, LG15, and LG17 frogs all have the MHC haplotypes *a* and *c*. However, the LG6 DNA has a 24-kb *Eco*RV fragment that is not shared by LG17. Furthermore, LG15 has a 24-kb *Pst* I fragment that is not shared by either LG6 or LG17. LG15 also lacks a 6-kb *Pst* I fragment that is present in LG6 and LG17. In each of these cases, frogs with identical MHC haplotypes have different C3 restriction fragment polymorphisms. In addition, two individuals, LG3

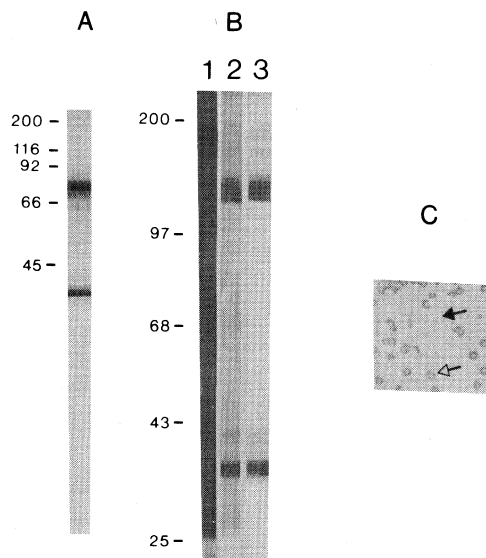


FIG. 1. Characterization of anti-*Xenopus* C3 antibody and *Xenopus* C3 clone. (A) Coomassie blue-stained SDS/polyacrylamide gel (22) of *Xenopus* C3 fragments eluted from zymosan. Markers at left indicate positions and $M_r \times 10^{-3}$ of standard proteins. (B) Immunoblots of *Xenopus* serum probed with rabbit anti-*Xenopus* C3 antibody (lane 1), antibody affinity-purified on zymosan particles having fixed *Xenopus* C3 α -chain fragments (lane 2), or antibody affinity-purified on clone C3-27 (lane 3). (C) Reactive C3-27 (open arrow) and unreactive (solid arrow) plaques with affinity-purified anti-*Xenopus* C3 antibody.

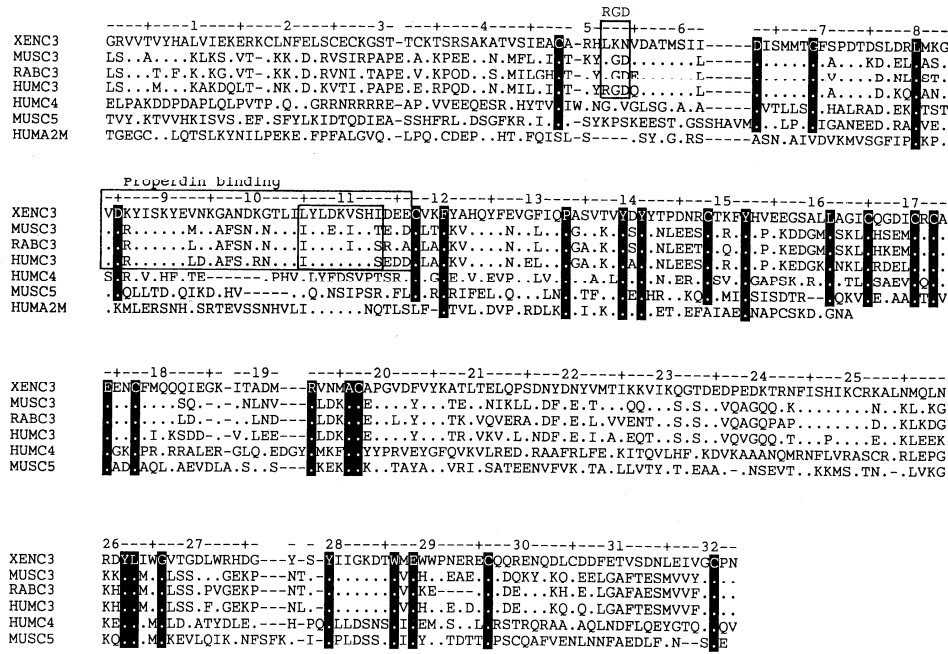


FIG. 4. Comparison of the deduced *Xenopus* C3 (XENC3) amino acid sequence with sequences of mouse C3 (MUSC3) (23), rabbit C3 (RABC3) (24), human C3 (HUMC3) (25), human C4 (HUMC4) (26), mouse C5 (MUSC5) (27), and human α_2 M (HUMA2M) (28). The numbering is from the *Xenopus* sequence. The first amino acid in each other case is as follows: mouse C3, 1342; rabbit C3, 405; human C3, 1342; human C4, 1403; mouse C5, 1322; human α_2 M, 1313. The box labeled "RGD" encloses the Arg-Gly-Asp sequence of human C3 and the corresponding sequences of the other C3 sequences. The boxes labeled "properdin binding" enclose the human C3 regions found to bind properdin (29). Identities with the *Xenopus* sequence are shown by dots, and gaps are shown by dashes. Residues shown in white-black type are conserved in all complement sequences aligned.

DISCUSSION

The initiation of the alternative pathway of complement activation, which occurs in the absence of antibody, relies on the slow but continuous activation of C3 (2). During complement activation the C3b fragment of C3 can be fixed on self and non-self surfaces, but in the case of self it is inactivated by widely distributed down-regulatory molecules (32). The alternative pathway may thus be a very ancient form of immunological defense, pre-dating the evolution of antibodies.

Xenopus C3 has the same subunit structure as mammalian C3 (7). This structure is also shared by the C3 from bird (33), reptile (34), and fish (35) but not by the C3 from lamprey, which has a three-subunit structure (36). C3b binds cell surfaces via its thioester site, and this property was utilized to purify *Xenopus* C3 bound to zymosan, which in turn was used to prepare a rabbit anti-*Xenopus* C3b antibody. This antibody was used to isolate an expression clone representing *Xenopus* C3 from a liver cDNA library. Liver is the major site of C3 synthesis in mammals (37). The anti-C3 antibody, which was affinity-purified on protein expressed by the C3-27 clone, recognized the same fragments of C3 on immunoblot strips as the antibody purified on C3 fragments fixed to zymosan (Fig. 1B, lanes 2 and 3). This verified that the clone indeed represents *Xenopus* C3. The M_r 115,000 protein represents the α' chain of C3b, and the M_r 34,000 protein represents the C-terminal end of the α chain of iC3b (7).

The sequence of *Xenopus* C3 has significant nucleotide and amino acid similarity with the corresponding sequence of

human (25), mouse (23), and rabbit (24) C3, the three species for which C3 sequence is available. Alignment of the *Xenopus* C3 sequence with these sequences shows clusters of sequence conservation. Although present, these conserved clusters are not as apparent when mammalian sequences are compared among themselves, presumably because the mammalian species from which C3 sequence is available are too closely related. One of these conserved clusters correlates quite closely with the region of C3b that mediates properdin binding. It is also interesting that the human RGD sequence is absent in *Xenopus* C3. The RGD sequence has been reported to be involved in iC3b binding to CR3 (30) and functions as a general attachment site of several adhesion molecules to their receptors (38, 39). Mammalian macrophages have CR3, as well as the more widely distributed C3b

Table 1. Amino acid conservation between *Xenopus* C3 and other related proteins

	% identity					
	Human C3	Mouse C3	Rabbit C3	Human C4	Mouse C5	Human α_2 M
<i>Xenopus</i> C3	48	50	49	25	28	22
Human C3		76	76	21	24	20
Mouse C3			78	22	25	20
Rabbit C3				22	24	18
Human C4					17	14
Mouse C5						13

Values are percentages of identities for all positions that are matched in the alignment shown in Fig. 3.

Pst I EcoRV
 LG: 3 15 17 6 3 15 17 6
 haplotypes : bd bc ac ac bd bc ac ac

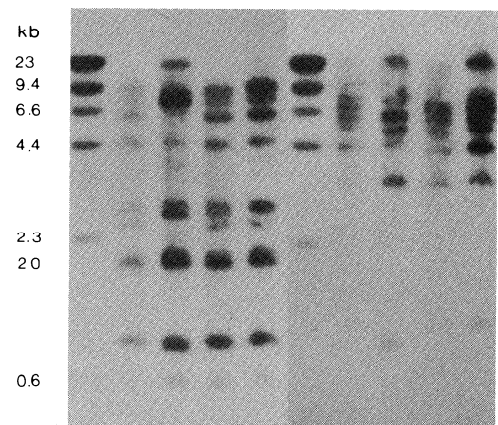


FIG. 5. MHC non-linkage of DNA from MHC-typed *X. laevis* \times *X. gilli* hybrid siblings. DNA was digested with *Pst* I (Left) or *EcoRV* (Right). Leftmost lane in each blot shows *Hind*III fragments of phage λ DNA used as size markers.

receptor, CR1 (1). Since *Xenopus* macrophages bind *Xenopus* C3b/iC3b-coated sheep erythrocytes (9), either this binding was via the *Xenopus* equivalent of CR1 or the binding of *Xenopus* iC3b to the CR3 receptor does not require the RGD sequence. The 20 amino acids extending to the C terminus of the human RGD sequence are highly conserved in all C3 sequences. Since these residues were present in the peptides used to show binding to CR3 (30), it may be these, rather than RGD, that are important for CR3 binding. The other extended regions of conservation with the other C3 sequences (amino acids 56–74, 170–180, 244–254, and 279–286) may be involved in binding of the other C3 ligands. C3 has many natural ligands besides properdin, some of which are factors I, H and B, C5, CR1, CR2, CR3, CR4, and membrane cofactor protein (MCP) (1, 3). Ligands must evolve to match each other, and the necessity for dual evolution is expected to make change at these sites slower. This seems to have been the case for the site of properdin binding.

The *Xenopus* sequence is 2–3% more similar to C4, C5, and α_2 M than are the other mammalian C3 sequences. Although this difference in similarity is small, it is present in all three sequences and suggests that *Xenopus* C3 has evolved more slowly than mammalian C3. The nucleotide alignment shows no stretches of sequence with sufficient nucleotide similarity expected to permit the isolation of this gene by hybridization with human probes. This may explain the inability to isolate a *Xenopus* C4 clone by low-stringency hybridization with human or mouse probes (D.G., unpublished observations).

C3, C4, C5, and α_2 M are structurally related and are thought to have arisen by gene duplication from an ancestral gene (40). The most primitive vertebrate, the lamprey, has "C3" with a subunit structure similar to mammalian C4, and it has been suggested to represent the ancestor molecule of higher vertebrate C3 and C4 (41). The C4 gene is in the MHC of *Xenopus* (42), humans, and mice (43), although complement proteins have not been found to interact in any way with MHC proteins. The C3 gene is not in the MHC of humans (44) or mice (45). The genes for C4 and the other complement components factor B and C2 have been said to reside in the MHC as a result of an accidental translocation (46). But these genes may have evolved within the MHC, and the C3 gene may have translocated out. To determine whether the C3 gene of *Xenopus* is linked to the *Xenopus* MHC, a Southern blot was done with DNA of a family of *X. laevis* \times *X. gilli* hybrid siblings. The polymorphisms observed did not cosegregate with the MHC alleles, and C3 is therefore not linked to the *Xenopus* MHC, as is the case with mammals. The translocation event that separated these duplicated genes must therefore have antedated the *Xenopus*/mammal divergence.

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- Lambris, J. D. (1988) *Immunol. Today* **9**, 387–393.
- Pangburn, M. K. & Müller-Eberhard, H. J. (1984) *Springer Semin. Immunopathol.* **7**, 163–192.
- Lambris, J. D. & Müller-Eberhard, H. J. (1986) *Mol. Immunol.* **23**, 1237–1242.
- Fujii, T., Sekizawa, A. & Katagiri, C. (1985) *Immunology* **56**, 743–750.
- Jurd, R. J. (1978) *Immunology* **34**, 389–396.
- Dlabac, V., Simeckova, J., Sima, P. & Hofmanova, B. (1983) *Dev. Comp. Immunol.* **7**, 783–784.
- Sekizawa, A., Fujii, T. & Katagiri, C. (1984) *J. Immunol.* **133**, 1436–1443.
- Kaidoh, T. & Gigli, I. (1987) *J. Immunol.* **139**, 194–201.
- Sekizawa, A., Fujii, T. & Tochinal, S. (1984) *J. Immunol.* **133**, 1431–1435.
- Becherer, J. D., Daoudaki, M. E. & Lambris, J. D. (1987) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **46**, 771 (abstr.).
- Ullrich, A., Shine, J., Chirgwin, J., Pictet, R., Tischler, E., Rutter, W. J. & Goodman, H. M. (1977) *Science* **196**, 1313–1319.
- Young, R. A. & Davis, R. W. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1194–1198.
- Huynh, T. V., Young, R. A. & Davis, R. W. (1985) in *DNA Cloning. A Practical Approach*, eds. Rickwood, D. & Hames, B. D. (IRL, Oxford), Vol. 1, pp. 49–78.
- Young, R. A. & Davis, R. W. (1983) *Science* **222**, 778–782.
- Towbin, H. T., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
- Chen, E. Y. & Seeburg, P. H. (1985) *DNA* **4**, 165–170.
- Needleman, S. & Wunsch, C. (1970) *J. Mol. Biol.* **48**, 444–453.
- Schwager, J., Grossberger, D. & DuPasquier, L. (1988) *EMBO J.* **7**, 2409–2415.
- Vogelstein, B. & Gillespie, D. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 615–619.
- Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13.
- Tack, B. F. (1983) *Springer Semin. Immunopathol.* **6**, 259–282.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 68–685.
- Wetsel, R. A., Lundwall, A., Davidson, F., Gibson, T., Tack, B. F. & Fey, G. H. (1984) *J. Biol. Chem.* **259**, 13857–13862.
- Kusano, M., Choi, N. H., Tomita, M., Yamamoto, K.-I., Migita, S., Sekiya, T. & Nishimura, S. (1986) *Immunol. Invest.* **15**, 365–378.
- deBruijn, M. H. L. & Fey, G. H. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 708–712.
- Belt, K. T., Carroll, M. C. & Porter, R. R. (1984) *Cell* **36**, 907–914.
- Wetsel, R. A., Ogata, R. T. & Tack, B. F. (1987) *Biochemistry* **26**, 737–743.
- Kan, C.-C., Solomon, E., Belt, K. T., Chain, A. C., Hiorns, L. R. & Fey, G. H. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 2282–2286.
- Daoudaki, M. E., Becherer, J. D. & Lambris, J. D. (1988) *J. Immunol.* **140**, 1577–1580.
- Wright, S. D., Reddy, P. A., Jong, M. T. C. & Erickson, B. W. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1965–1968.
- Kobel, H. R. & Du Pasquier, L. (1975) *Immunogenetics* **2**, 87–91.
- Atkinson, J. P. & Farries, T. (1987) *Immunol. Today* **8**, 212–215.
- Kai, C., Yoshikawa, Y., Yamanouchi, K. & Okada, H. (1983) *J. Immunol.* **130**, 2814–2820.
- Eggersten, G., Lundwall, A., Hellman, U. & Sjoquist, J. (1983) *J. Immunol.* **131**, 1920–1923.
- Nonaka, M., Iwaki, M., Nakai, C., Nozaki, M., Kaidoh, T., Natsuume-Sakai, S. & Takahashi, M. (1984) *J. Biol. Chem.* **259**, 6327–6333.
- Nonaka, M., Fujii, T., Kaidoh, T., Natsuume-Sakai, S., Yamaguchi, N. & Takahashi, M. (1984) *J. Immunol.* **133**, 3242–3249.
- Alper, C. A., Johnson, A. M., Birch, A. G. & Moore, F. D. (1969) *Science* **163**, 286–288.
- Ruoslahti, E. & Pierschbacher, M. D. (1986) *Cell* **44**, 517–518.
- Simmons, D., Makgoba, M. W. & Secd, B. (1988) *Nature (London)* **331**, 624–627.
- Sottrup-Jensen, L., Stepanik, T. M., Kristensen, T., Lonblad, P. B., Jones, C. M., Wierzbicki, D. M., Magnusson, S., Domdey, H., Wetsel, R. A., Lundwall, A., Tack, B. F. & Fey, G. H. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 9–13.
- Nonaka, M., Fujii, T., Kaidoh, T., Natsuume-Sakai, S., Yamaguchi, N. & Takahashi, M. (1984) *J. Immunol.* **133**, 3242–3249.
- Nakamura, T., Sekizawa, A., Fujii, T. & Katagiri, C. (1986) *Immunogenetics* **23**, 181–186.
- Alper, C. A. (1981) in *The Role of the Major Histocompatibility Complex in Immunobiology*, ed. Dorf, M. E. (Garland STPM, New York), pp. 173–220.
- Whitehead, A. S., Soloman, E., Chambers, S., Bodmer, W. F., Povey, S. & Fey, G. H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5021–5025.
- Ferreira, A. & Nussenzweig, V. (1975) *J. Exp. Med.* **141**, 513–517.
- Klein, J. & Figueroa, F. (1986) *CRC Crit. Rev. Immunol.* **6**, 295–386.

MONOVALENT LIGANDS OF COMPLEMENT RECEPTOR 2 INHIBIT WHEREAS POLYVALENT LIGANDS ENHANCE ANTI-Ig-INDUCED HUMAN B CELL INTRACYTOPLASMIC FREE CALCIUM CONCENTRATION¹

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We have performed experiments to investigate the role of ligands for complement receptor 2 (CR2) in human B cell activation. Flow microfluorimetry was used to assess changes in free intracytoplasmic calcium concentration $[Ca^{2+}]_i$ in indo-loaded B cells, immediately after exposure to anti- μ antibody and to monovalent or polyvalent CR2 ligands. As monovalent ligands we used the C3d fragment and synthetic C3 peptides (peptides P14, residues 1201-1214, and P28, residues 1187-1214). As polyvalent ligands we used i) an intact monoclonal mouse anti-CR2 antibody (HB5) and its $F(ab')_2$ fragment, ii) tetravalent P13 ((residues 1202-1214) 4-template), and iii) P28 conjugated to BSA (molar ratio 5/1). Anti-CR2 antibody HB5, tetravalent P13, and P28 conjugated to BSA, enhanced the ability of $F(ab')_2$ fragments of the IgG fraction of goat anti-human μ antibody to increase human B cell $[Ca^{2+}]_i$. In contrast, the monomeric CR2 ligands C3d and P28 inhibited the anti- μ -induced increase in human B cell $[Ca^{2+}]_i$. Multivalent P13, P28, and the HB5, by themselves, did not affect B cell $[Ca^{2+}]_i$. These experiments suggest that the valence of the CR2 ligands is crucial for the nature (synergistic vs antagonistic) of the message transmitted through the CR2.

CR2³ is a 140-kDa glycoprotein (1-3) that specifically binds iC3b, C3dg, and C3d fragments of C3 (4, 5) and the EBV envelope protein gp350/220 (6, 7). CR2 is expressed primarily by B cells, although it has been found on other cell types (5, 8).

CR2 binds a site on C3 that is composed of residues 1205-1214 of the C3 sequence (9). Synthetic C3 peptides P14 (residues 1201-1214) and P28 (residues 1187-1214) bind to CR2 (9, 10) and can be used as ligands in functional assays. A number of murine mAb that bind human

CR2, including the murine mAb HB5, have been produced (4, 8).

CR2 has been considered to play a role in B cell differentiation and proliferation. Polyclonal and monoclonal antibodies to CR2 (11-13) as well as particle bound C3d (14) have been shown to enhance B cell responses in different systems. More recently, HB5 has been found to act synergistically with μ -chain-specific antibody to increase B cell intracytoplasmic free calcium $[Ca^{2+}]_i$ (15). Monomeric fluid phase C3d, in contrast, has been found to inhibit murine B cell proliferation (14). P14 and P28 peptides inhibit the maturation of murine B cell progenitors (16) while they support the growth of CR2-binding EBV lymphoblastoid B cell lines (10).

Cross-linking of B cell surface Ig induces hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C to inositol trisphosphate and diacylglycerol. Inositol trisphosphate causes the release of intracellular calcium from endoplasmic reticulum (17-21). $[Ca^{2+}]_i$ can be accurately measured in isolated B cells using the Ca^{2+} -dependent indicator indo-1 (21, 22).

In this study we used the anti- μ -induced increase in $[Ca^{2+}]_i$, an early event in B cell activation, to study the role of CR2 in B cell function. We found that CR2-mediated modulation of B cell activation is ligand-valency-dependent, in that monomeric ligands inhibit the anti- μ -induced increase in $[Ca^{2+}]_i$ whereas polyvalent CR2 ligands enhance the ability of anti- μ to increase $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Cells. Peripheral blood MNC were obtained from heparinized blood by Ficoll-Hypaque density centrifugation. Human spleen cells were obtained through the Tissue Procurement Service of the Clinical Center of the National Institutes of Health.

Reagents. Affinity-purified $F(ab')_2$ fragments of the IgG fraction of goat anti-human μ (purchased from Jackson ImmunoResearch Laboratories, West Grove, PA) were used to stimulate B cells. PE-labeled monoclonal mouse IgG1k anti-human CD20 antibody was purchased from Becton Dickinson (Mountain View, CA). PE-labeled Leu-4, Leu-M3, Leu-7, and Leu-11 (Becton Dickinson) were used in some experiments. HB5 mouse IgG2ak anti-CR2 was obtained from the American Type Culture Collection, Rockville, MD. $F(ab')_2$ fragments of HB5 were prepared by pepsin digestion and separated from undigested IgG2ak molecules by absorption with protein A Sepharose (Pharmacia, Piscataway, NJ). Purity was demonstrated by SDS-PAGE analysis. $F(ab')_2$ fragments of the IgG fraction of goat anti-murine IgG antibody were purchased from Cappel-Cooper Biomedicals (Malvern, PA). $F(ab')_2$ fragments of goat anti-human- μ and/or HB5 antibodies were bound onto cyanogen bromide-activated Sepharose (Pharmacia) according to the provided instructions.

C3 was prepared from human plasma and C3d was prepared by digestion as previously described (23). C3-derived CR2-binding pep-

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³ Abbreviations used in this paper: CR, complement receptor; $[Ca^{2+}]_i$, intracytoplasmic free calcium concentration; MNC, mononuclear cells; PE, phycoerythrin.