

## C3 SYNTHETIC PEPTIDES SUPPORT GROWTH OF HUMAN CR2-POSITIVE LYMPHOBLASTOID B CELLS<sup>1</sup>

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The nature of CR type 2 (CR2)-ligand interactions which leads to the activation of human B cells was analyzed by using synthetic peptides and CR2-positive cell lines. The third component of C (C3) supported the growth of human lymphoblastoid B cells in serum-free medium containing human transferrin. This effect was inhibited by an antibody to C3d (mAb 130) which specifically inhibits C3d binding to CR2, but not by other anti-C3 mAb. Synthetic peptides corresponding to the CR2-binding site on C3d, P28 (residues 1187-1214) or multivalent P13 ((1202-1214)<sub>4</sub>-template), supported the proliferative response of CR2-positive human lymphoblastoid lines in a similar way as C3 and this response could be inhibited by the anti-CR2 mAb OKB7. The proliferative response to C3 or peptides was dose dependent and a 60-fold higher concentration of P28 peptide was required to induce the same level of proliferation as C3. This stimulation of growth was observed only on CR2 expressing cell lines Raji and Daudi, and not on the CR2-negative Burkitt lymphoma cell line Rael and the monocytic cell line U937. In contrast to the stimulatory effect of P28 and P13-template, monomeric P14 (1201-1214) was not able to support the growth of these cell lines. This peptide, however, inhibited the proliferative response of the CR2-positive lines to C3, P28, and multivalent-P13, thus indicating that cross-linking of the CR2 receptor is necessary for B cell proliferation. Another peptide, E12 (from glycoprotein (GP)350, the major EBV outer membrane GP) which shows a high degree of similarity with P14, also inhibited the proliferative response of Raji cells, suggesting that this segment on GP350 is involved in the interaction of EBV with CR2. The possibility of using the above peptides as well as other peptides with "tailor-made" structure in studying the multifunctional role of C3 is discussed.

The EBV/C3d<sup>3</sup> receptor or CR2, a member of the family of C3b/C4b binding proteins (1, 2), is a 140,000 Da glycoprotein (3-5), with binding specificity for iC3b, C3dg, and C3d fragments of C3 (6-8) as well as for the

EBV envelope protein GP350/220 (9, 10). It is expressed on human B cells (11), human pharyngeal epithelial cells (12), human follicular dendritic cells (13), cervical epithelium (14), thymocytes (15), and human T cell lines (16, 17), but absent from peripheral T cells, NK cells, and cells of the erythroid and myeloid lineages (18).

The CR2-binding site on C3 has been previously localized to residues 1205-1214 of the C3 sequence (19). The CR2 binding site on GP350 has not yet been identified; however, two regions of amino acid similarity were found in the GP350 and C3d coding sequences (9, 10, 20) and it was suggested that they may represent CR2-binding sites of GP350. The nature of the receptor-binding site to which EBV and C3d binds is not known, and it is also not clear whether both ligands react with the same sites. In a recent study, however, by using anti-idiotypic anti-GP140 antibodies, it was suggested that the binding sites for C3d and EBV on the CR2 are distinct (21).

CR2 has long been implicated in the control of B cell responses. Cross-linking of CR2 on the cell surface by polyclonal anti-CR2 F(ab)<sub>2</sub>' (22), anti-CR2 mAb (23, 24), or particle-bound C3d (23, 25) have been shown to enhance B cell proliferation. In addition, cross-linking of CR2 by a anti-CR2 mAb enhanced the anti-IgM-induced increase of intracellular free calcium concentration (26). Recently, it has been shown that C3 (27) or mAb to CR2 (17) can induce the growth of Raji cells. Although the role of CR2 in the regulation of B cell responses is well documented, the exact mechanisms that operate are unknown. The elucidation of the mechanism that operates in these CR2-mediated B cell responses is further complicated by the fact that its ligand, C3d, reacts with several serum proteins and cell surface receptors other than CR2 (28-30).

In the present experiments we made use of synthetic peptides with monovalent or multivalent nature in order to mimic the interaction between C3d and CR2 receptor and to study the mechanism(s) that leads to B cell activation and growth. As a test system we used the previously established CR2-mediated growth of human lymphoblastoid cell lines (27). Synthetic peptides containing the CR2-binding site on C3d support the growth of EBV-transformed lines under serum free conditions to the same extent as native C3. By using a multivalent synthetic peptide, we demonstrated that cross-linking of CR2 is necessary to support B cell growth. In addition, we showed that the synthetic peptide E12 from GP350, the major EBV outer membrane GP, inhibits the C3 supported B cell growth, thus suggesting that the segment of GP350 represented by this peptide is involved in the interaction between EBV and CR2.

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<sup>3</sup> Abbreviations used in this paper: C3, third component of C; CR2, receptor for the iC3b, and C3d fragments of C3; GP, glycoprotein.

## MATERIALS AND METHODS

**C proteins.** Human C3 was isolated from EDTA plasma as previously described (31).

**Synthetic peptides.** All peptides (Fig. 1) were synthesized by the standard solid phase method of Merrifield and Stewart et al. (33) by using an Applied Biosystems model 420A automated peptide synthesizer. The peptides were synthesized on a *p*-methylbenzhydrylamin resin, by using *t*-butyloxycarbonyl-protected amino acids (Bachem). The couplings were performed in dimethylformamide/dichloromethane by using dicyclohexylcarbodiimide or 1-hydroxybenzotriazole as coupling agents followed by neutralization with *N,N*-diisopropylethylamine and trifluoroacetic acid (40%) for deprotection. The coupling efficiency was followed after each cycle by the Kaiser-ninhydrin test (34). The P13-template was synthesized according to the principle of Mutter et al. (35). The template was synthesized first on a *t*-butyloxycarbonyl-Gly-*p*-methylbenzhydrylamine resin by using butyloxycarbonyl-Lys(F-moc)-OH for cycles 1, 3, 6, and 8, whereas the other couplings were performed using *t*-butyloxycarbonyl-Lys-(Cl-Z)-OH derivatives. After completion of the synthesis the F-moc protected side chains were deprotected by using 10% pyridine in dimethylformamide and then four P13 (Fig. 1) peptide chains were synthesized on the template-resin by using *t*-butyloxycarbonyl-protected amino acids. The four P13 peptides were synthesized, starting from the C terminus, on the  $\epsilon$ -NH<sub>2</sub> group of lysines 1, 3, 6, and 8 of the template. The final products were cleaved from the resin, using high anhydrous HF in the presence of anisole at a ratio of HF/anisole/peptide resin of 10/1/1 for 1 h at 4°C (36) and after washing the resin with cold ether, the peptide was extracted with 10% acetic acid. The crude peptides were purified by gel filtration on a Sephadex G-15 column equilibrated with 2% acetic acid followed by reverse-phase HPLC on a C<sub>18</sub> (Vydac) column by using a 60-min gradient of 0 to 80% acetonitrile containing 0.1% trifluoroacetic acid. The purity and composition of the peptides were confirmed by TLC and amino acid analysis by using an Applied Biosystems 420A derivatizer with an on-line 130A analyzer. The peptides were sequenced by Edman degradation by using an Applied Biosystems 740A gas phase sequencer with an on line 120A PTH analyzer and a 900A data handling system. The T4(α<sub>II</sub>) peptide (35) was kindly provided by Prof. M. Mutter, Department of Organic Chemistry, University of Basel. All peptides were dialysed against PBS (pH 7.2) and filtered through 0.2-μm filters (Millipore) before use in culture.

**Antibodies.** The anti-CR2 mAb (HB-5)-producing cell line was obtained from the American Type Culture Collection (Rockville, MD) and the antibody was purified from ascites on a protein A column. The anti-CR2 mAb OKB7 was purchased from Ortho Diagnostics (Raritan, NJ). The two anti-C3c mAb C3-9 and 133H11 were generously provided by Dr. E. Hack and Dr. J. Alsenz, respectively.

**Cell lines.** The cell line Raji, a CR2-positive B cell line derived from Burkitt's lymphoma, and the monocytic cell line U937 were

obtained from the American Type Culture Collection. The lymphoblastoid lines Daudi, Ramos, and Rael were given by Dr. Eitan Yefenof (Department of Immunology, Hadassa Medical School, Jerusalem).

**Cell cultures.** All cell lines were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin (200 IU/ml), streptomycin (100 μg/ml), and 10% heat-inactivated FCS (GIBCO) at 37°C in a 5% CO<sub>2</sub> incubator. For the cell growth experiments the medium was Iscove's modified Dulbecco's medium supplemented with 2 mM L-glutamine, penicillin (200 IU/ml), streptomycin (100 μg/ml), and 10 μg/ml human transferrin (Sigma). The stimulation of lymphoblastoid cell growth in the above serum-free medium by C3 or C3 synthetic peptides was tested by using a proliferation assay. The cells were washed four times in the serum-free medium, resuspended in the same medium, seeded in triplicate in 96-well microtiter plates at a concentration of 7.5 × 10<sup>3</sup>/ml (200 μl total volume/well), and cultured in the presence or absence of C3 or synthetic peptides at 37°C for different time intervals. Proliferation was assessed by measuring DNA synthesis of cells after adding 0.5 μCi [<sup>3</sup>H]TdR (sp. act. 5 Ci/mmol, Amersham, United Kingdom) and measuring the radioactivity incorporated after 18 h. In the inhibition experiments competitor synthetic peptides or mAb against different C3 fragments or CR2 were added to the cultures together with suboptimal concentrations of C3 or synthetic peptide.

## RESULTS

**Growth supporting effect of human C3 or C3 synthetic peptides on CR2 positive human lymphoblastoid B cells.** In order to assess the growth-supporting effect of C3 synthetic peptides on the Burkitt tumor cell line, Raji, the cells were cultured in a serum-free medium containing human transferrin (10 μg/ml), and seeded at concentrations of 7.5 × 10<sup>3</sup> cells/ml (200 μl/well). [<sup>3</sup>H]TdR (0.5 μCi) was added to each culture and the radioactivity incorporated was measured after 18 h and taken as indicator of DNA synthesis of the cultured cells. The stimulation of DNA synthesis by C3 or synthetic peptide (see Fig. 1) was measured in the lymphoblastoid cell cultures at time intervals of 2 days (Fig. 2). Maximum incorporation of [<sup>3</sup>H]TdR in cultures containing 70 nM C3 or synthetic peptide (P28, 3.8 μM; P13-template, 1.9 μM) was reached at day 8 of incubation. The C3 synthetic peptides P28 and P13-template (Fig. 1) had the same

PEPTIDE	RESIDUE	SEQUENCE
P28	1187-1214 (C3)	KFLTTAKDKNRWEDPGKQLYNVEATSYA
P14	1201-1214 (C3)	PGKQLYNVEATSYA
I17	91-107 (IgVA2)	ALWFRNHVFVGGTKVT
OVA	173-196 (OVA)	VLVNAIVFKLWLEKAFKDEDTQAM
E12	19-32 (GP350)	TGEDPGFFNVEI
P13-template	(1202-1214) <sub>4</sub> -template	GKQLYNVEATSYA ← K GKQLYNVEATSYA ← K GKQLYNVEATSYA ← P GKQLYNVEATSYA ← G GKQLYNVEATSYA ← K GKQLYNVEATSYA ← E GKQLYNVEATSYA ← K GKQLYNVEATSYA ← G
T4-(4α <sub>II</sub> )		LKLANALATAAD ← K LKLANALATAAD ← K LKLANALATAAD ← P LKLANALATAAD ← G LKLANALATAAD ← K LKLANALATAAD ← E LKLANALATAAD ← K LKLANALATAAD ← G

Figure 1. Amino acid sequences of the synthetic peptides used in this study. The numbering of amino acid residues of C3 peptides is based on the predicted sequence of C3 (43) after subtracting the signal peptide sequence.

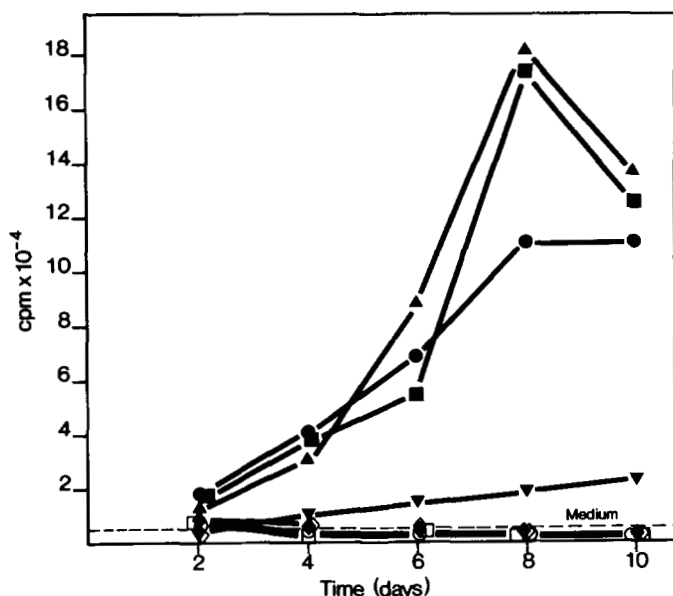


Figure 2. Time course of Raji cell growth in the presence of C3 or synthetic peptide. Raji cells ( $7.5 \times 10^3$ /ml) were cultured in serum-free medium containing human transferrin in the presence of 70 nM C3 (■), 3.8  $\mu$ M P28 (●), 1.9  $\mu$ M P13-template (▲), 8  $\mu$ M P14 (◆), 1.9  $\mu$ M T4( $\alpha_{II}$ ) (◇), 6.4  $\mu$ M I1F (▼), 4.5  $\mu$ M OVA (○), and 9.3  $\mu$ M E12 (□). Stimulation of growth was measured by [ $^3$ H]TdR incorporation.

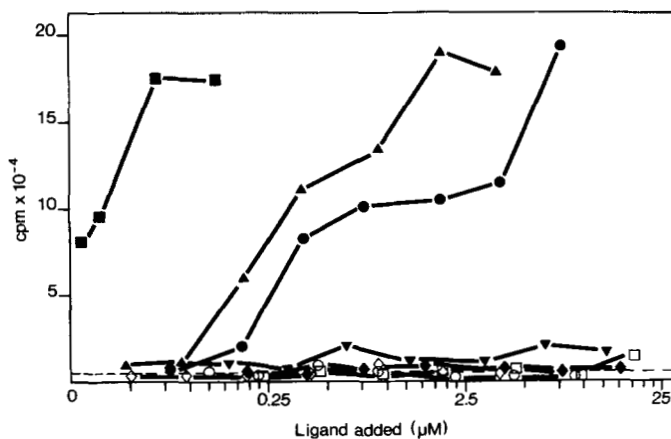


Figure 3. Effect of C3 or synthetic peptides on Raji cell growth as assessed by incorporation of [ $^3$ H]TdR. Raji cells were cultured for 6 days in serum free medium containing human transferrin (10  $\mu$ g/ml) at a concentration of  $7.5 \times 10^3$ /ml in the presence of different amounts of C3 (■), P28 (●), P13-template (▲), P14 (◆), E12 (□), T4( $\alpha_{II}$ ) (◇), OVA (○), or I1F (▼).

stimulatory effect on the growth of Raji cells as C3, whereas P14, E12, and the unrelated control peptides T4( $\alpha_{II}$ ), OVA, and I1F did not support the growth of these cells (Fig. 2). In the absence of C3 or the stimulatory peptides the incorporation of [ $^3$ H]TdR was marginal and the cells died after 2 days in culture. The degree of stimulation by C3 or synthetic peptide was dependent on the cell concentration in the cultures, as cells seeded lower than  $3.75 \times 10^3$  cells/ml did not grow in the presence of C3 or peptides (data not shown).

The proliferative response of Raji cells to C3 or C3 synthetic peptides under these conditions was dose dependent (Fig. 3). The amount of P28 or P13-template required to obtain the same effect as with C3 was about 60- and 30-fold higher than that of C3, respectively.

To confirm that the growth supporting effect of Raji cells by C3 was due to a specific receptor-ligand interac-

tion, different anti-C3 mAb were tested for their ability to inhibit the C3-induced growth. Figure 4 shows that the mAb 130, which recognizes the CR2-binding site on C3 inhibited the growth-supporting effect of C3 on Raji cells. None of the other mAb tested which recognize other epitopes on C3 inhibited this effect of C3 (Fig. 4). Furthermore, incubation of the cells with antibody OKB7 which blocks binding of C3d to CR2, inhibited the growth supporting affect of P13-template (Fig. 5). The mAb HB-5 which reacts with a different epitope on CR2 than OKB7 had no inhibitory effect.

The growth-supporting effect of C3 or C3 synthetic peptides was observed only on those lymphoblastoid B cell lines that were CR2 receptor positive (Table I). The EBV-transformed lines, Raji and Daudi, which are CR2 positive proliferated in response to C3, whereas the monocytic U937 cells divided slowly under the same conditions and C3 or the synthetic peptides at different concentrations (1 to 25  $\mu$ g/ml) had no effect on cell proliferation (Table I). Rael, an EBV-carrying Burkitt lymphoma which is C3dR negative died within 2 days in serum-free culture in the presence of C3. The observation that only C3dR positive cell lines were stimulated to proliferate in the presence of C3 or C3 synthetic peptide supports the above results which indicate that CR2 is involved in this C3 supported growth.

*Inhibition of the growth-supporting effect of C3 or C3 synthetic peptides on the growth of Raji cells by monomeric peptides.* The results presented in Figures 2 and 3 indicated that cross-linking of CR2 receptors may be necessary for supporting the growth of Raji cells, since multivalent P13-template but not monomeric P14 was able to support the growth of Raji cells. To test this possibility, monomeric P14 and the peptide E12 from GP350, which shows high amino acid similarity with P14, were added in culture at different concentrations and tested in their ability to inhibit the proliferative response of Raji cells to a suboptimal concentration of C3 (2.8 nM) or multivalent P13 (75 nM). Figure 6 shows that P14 as well as E12 inhibited the growth of Raji cells supported by either C3 or P13-template. The inhibition of P28 growth supporting effect by P14 was similar to that observed with C3 or P13-template (data not shown).

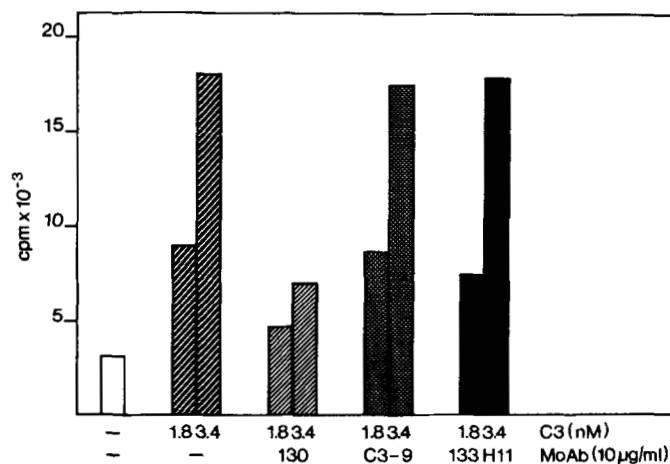


Figure 4. Inhibition of C3 supported Raji cell growth by anti-C3 mAb. Raji cells were cultured for 6 days in serum-free medium at a concentration of  $6 \times 10^3$  cells/ml in the presence of C3 alone or C3 and 10  $\mu$ g/ml C3 mAb 130, C3-9, and 133H11.

Figure 5. Inhibition of P13-template supported Raji cell growth by anti-CR2 mAb. Raji cells were cultured for 6 days in serum-free medium at a concentration of  $6 \times 10^3$  cells/ml in the presence of P13-template alone or P13-template and 10  $\mu$ g/ml anti-CR2 mAb OKB7 and HB-5.

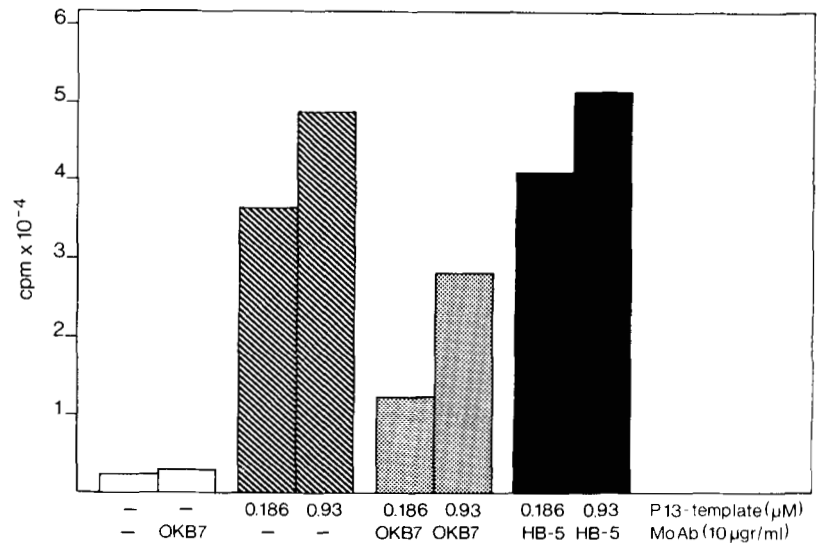


TABLE I

Comparison of the growth supporting effect of C3 or peptides on different human cell lines

Cell lines	<sup>3</sup> H]TdR Incorporation (cpm × 10 <sup>-4</sup> ) in Presence of:					
	C3	P28	P13-template	P14	OVA	Control
Raji	10.6	11.2	12.9	1.4	1.6	1.5
Daudi	11.5	10.3	10.9	1.6	1.5	1.8
Rael	1.1	1.2	1.1	1.0	1.5	1.2
U937	22.5	21.7	20.6	23.9	22.5	23

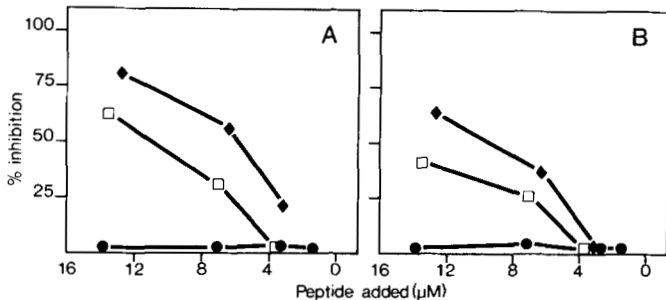


Figure 6. Inhibition of the stimulatory effect of C3, and P13-template on the growth of Raji cells by monomeric peptides. Raji cells were cultured in serum free medium at a concentration of  $6 \times 10^3$  cells/ml in the presence of 2.8 nM of C3 (A), or 75 nM P13-template (B) together with varying concentrations of monomeric P14 (◆), E12 (□), or OVA (○). Inhibition of proliferation was measured after 6 days of culture and expressed as percent reduction of cpm in cultures with competitor compared with those without.

## DISCUSSION

C activation via the classical or the alternative pathway by cell surfaces or immunocomplexes leads to the generation of two types of effector molecules, those that are fluid phase or bound to surfaces or immunocomplexes. Among the fragments generated, the C3 fragments can bind to six distinct cell surface receptors and mediate various biologic responses (for review, see Refs. 37 and 38). The existence of C3 fragments in two different forms (surface bound vs fluid phase), the multiple interaction of C3 and possible simultaneous interaction of the same fragment with different receptors, as well as the processing of natural C3 fragments during cell culture makes the use of these fragments somewhat problematic in studying the function of the individual receptors.

This study was undertaken to investigate the nature of CR2 ligands which support the growth of human lym-

phoblastoid B cell lines by using different forms of synthetic peptides, multivalent vs monomeric. We tested the effect of human C3 and C3 synthetic peptides on the growth of human lymphoblastoid B cells that were maintained in serum-free media containing human transferrin. Growth was assessed by measuring the increase of [<sup>3</sup>H]TdR incorporation in DNA and by observing the morphology of the cells in culture. The results show that C3 or synthetic peptides are able to support growth of CR2-positive cell lines which in the absence of C3 or synthetic peptide die within 2 days under these culture conditions. The growth-supporting effect of C3 or synthetic peptides is dose dependent, reaching its maximum after 6 to 8 days. The amount of synthetic peptide required to obtain the same effect as C3 was consistent with the data obtained from binding studies by using <sup>125</sup>I-labeled peptides (19). The observation that mAb 130 but not other anti-C3 mAb inhibits the C3-dependent proliferation and that only the CR2-binding peptides support the growth of CR2-positive cell lines, as well as the inhibition of this effect by OKB7, clearly demonstrate the involvement of CR2 in the growth of these cell lines.

The finding that multivalent P13, but not monomeric P14, supports the growth of Raji cells under these conditions indicates that cross-linking of CR2 is a necessary signal for the stimulation of proliferation of these cells. This is in agreement with previous results showing that cross-linking of CR2 by either anti-CR2 antibodies or aggregated C3-enhanced B cell proliferation (23, 25, 39). A recent report, however, suggested that monomeric C3 has a mitogenic effect on the growth of Raji cells (27). In those studies the nature of monomeric C3 was tested before addition to the culture and one cannot exclude the possibility that C3 gets modified (aggregated) during the culture. The modification of C3 during the culture is necessary in order to exert its effect. Native C3 does not react with CR2 and in order to bind to CR2 has to be either cleaved by cell derived proteases (40) or undergo conformational changes, e.g., by aggregation.

The stimulatory effect of the synthetic peptide P28, which contains the P14 sequence, but is extended 14 amino acids toward the amino terminus, can be explained by either proposing that the hydrophobic nature of its amino terminus (5 hydrophobic amino acids) results in

aggregation of P28 or that this N terminus segment contains an effector site necessary for this growth-supporting effect. A two-site interaction has also been proposed for the interaction of C fragment C5a with its receptor (41). An alternative explanation could be that P28 as well as P13-template assume a more favorable conformation than P14 for interacting with CR2 (e.g., bind with higher affinity).

Because both GP350/220 and the C3d fragment of C3 bind CR2 (9, 10) and the anti-CR2 mAb inhibits both EBV and C3d binding (42), it has been proposed that the two proteins may have similar domains which interact with the same binding site on CR2. Two homologous sites have been noted from the comparison of the predicted amino acid sequences of EBV and C3d (9, 10, 20, 43). Our results from the inhibition experiments by using monomeric peptides, P14 and E12, indicate that the stretch composed of amino acids 19–30 of the GP350 sequence (44, 45) is a CR2-binding site on GP350. The peptides P14 and E12 showed similar inhibitory activity although their primary sequences show a weak similarity, 5 identical amino acids out of 10 after the introduction of a gap. This suggests that the C3d and GP350 molecules assume a similar conformation in the CR2 binding area. The interaction of GP350 with CR2 via this site has also been recently shown in another study by using the EDPGFFNVE peptide (amino acids 21–29 of GP350) and direct binding assays (46). Further evidence of this segment reacting with CR2 comes from a recent study which showed that a deletion mutant of GP50/220 missing the VE amino acids (amino acid 28, 29 of GP350) failed to bind CR2 (47). The involvement of this segment of GP350 in binding to CR2, however, does not necessarily suggest that it is the only site through which EBV interacts with its receptor.

In addition to the data presented and discussed above, several conclusions can be drawn with respect to these synthetic peptides. Earlier studies have shown that it is possible to synthesize different types of templates with the attached peptide(s) in different orientations and different ability to stabilize the secondary structure of this peptide(s) ("tailor made" peptides) (35, 48). This suggests that it is possible to synthesize peptides with different specificities e.g., bind both CR1 and CR2 or CR2 and CR3 and so on, or with different properties, e.g., multivalent, as in case of P13-template (possibly mimicking surface bound C3 fragments). Because the binding sites on C3 for several of the CR and other C components have recently been identified (37, 48), the construction of such peptides may be instrumental in understanding the role of different C3 fragments in immunoregulation.

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