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Epstein Barr virus/complement C3d receptor is an interferon α receptor

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Interferon α contains a sequence motif similar to the complement receptor type two (CR2/CD21) binding site on complement fragment C3d. Antibodies against a peptide with the CR2 binding sequence on C3d react with a peptide carrying the IFNα CR2 binding motif (residues 92-99) and with recombinant IFNα. The IFNα-derived peptide, as well as recombinant IFNα, inhibits C3bi/C3d interaction with CR2 on the Burkitt lymphoma Raji. The direct interaction of IFNα and CR2 is inhibited by polyclonal anti-IFNα, anti-CR2 and anti-C3d peptide antibodies as well as by C3bi/C3d, EBV coat protein gp350/220 and IFN but not by IFNγ. [^{125}I]IFNα binding to Raji cells is inhibited by polyclonal anti-IFNα and anti-CR2 antibodies, by peptides with the CR2 binding motif and partially by C3bi/C3d. Monoclonal anti-CR2 antibody HB5, but not OKB-7, blocks IFNα binding to Raji cells. CR2 or CR2-like molecules may therefore be the major IFNα receptors on B lymphocytes.

Key words: lymphocytes/EBV/C3d receptor/Interferon α receptor/receptor binding motif

Introduction

Interferons are highly active intercellular mediators that induce viral resistance, inhibit cellular proliferation, and modulate immune responses (Pestka *et al.*, 1987). Interferons are classified into three antigenically and functionally different groups, designated IFNα, IFNβ and IFNγ. The beta and gamma IFNs are each encoded by a single gene, whereas the alpha IFNs are a family of at least 23 different members that show a high degree of sequence identity at both the nucleotide and amino acid levels (Weissmann and Weber, 1986). Currently, recombinant interferons are used in the treatment of a number of viral and malignant diseases (Strander, 1986). IFNα in particular, has been used to treat Hairy cell leukemia, non Hodgkin's lymphoma (Strander, 1986), hepatitis (Davis *et al.*, 1989; Di Bisceglie *et al.*, 1989), and it has shown success in the treatment of Kaposi's sarcoma and human immunodeficiency virus (HIV) infection (DeWit *et al.*, 1988; Lane *et al.*, 1988).

Interferons interact with cell surface receptors (Aguet, 1980; Mogensen and Bandu, 1983). Previous studies have suggested that human IFNα and β share a common receptor (Merlin *et al.*, 1985), while IFNγ interacts with a different receptor (Aguet *et al.*, 1988). Recently, a receptor that binds IFNαB but not other IFNα subtypes has been characterized on Daudi cells, a lymphoblastoid B cell line (Uze *et al.*, 1990). IFNα has two, probably related, effects on B lymphocytes: it inhibits both B cell proliferation and infection with Epstein Barr virus (EBV) (Pestka *et al.*, 1987; Lotz *et al.*, 1985). The receptor for EBV is complement receptor type 2 (CR2, CD21) and receptor binding is mediated by a specific site on EBV coat protein gp350/220 (Fingeroth *et al.*, 1984; Nemerow *et al.*, 1989). CR2 is also the receptor for proteolytic activation products of complement component C3, C3bi/C3d (Iida *et al.*, 1983; Weis *et al.*, 1984; Tedder *et al.*, 1984). The CR2 binding motif on C3d has been defined (Lambris *et al.*, 1985) and shows sequence similarity to the CR2 binding site on gp350/220. We have identified a sequence motif on IFNα (amino acids 92-99) that is similar to the CR2 binding site on complement fragment C3d. Computer analysis of the secondary structure of the C3d and IFNα sequences predicts that these motifs may be organized as surface loops followed by the starts of α-helices and that their three-dimensional structure may be similar. In this report, we show that the region composed of residues 92-99 (Figure 1A) on IFNα, conserved in most IFNα subtypes (Figure 1B), is a binding site for complement receptor type 2. Hence, CR2 or possibly a subpopulation of CR2 molecules may function as IFNα receptors on B lymphocytes.

Results

IFNα contains a CR2 binding site motif

To examine whether amino acids 92-99 on IFNα (the '92-99' motif, Figure 1A) specify a structural determinant similar to the CR2 binding domain on C3d we tested whether antibodies raised against one determinant would crossreact with the other. The IgG fraction of a rabbit antiserum raised against a synthetic peptide, QLYNVEATS (pC3d; corresponding to the CR2 binding site on C3d) (Lambris *et al.*, 1985) recognizes pC3d and the IFNαA derived peptide QLNDLEACV (pIFNα) specifically, but not an unrelated peptide, GRGDESP (pFib), derived from fibronectin (Figure 2A). When the IgG fraction from a preimmune serum was used, no binding was detected. Although the anti-pC3d antibodies reacted less well with pIFNα than with C3d, this result suggests that both peptides express at least one common epitope. To determine whether this epitope is recognized in the context of the entire molecule, an ELISA was carried out using C3bi/C3d and purified recombinant IFNαA. Figure 2B shows that the anti-pC3d antibodies bind C3bi/C3d and IFNαA but not fibronectin.

pIFNα mimics the activity of pC3d and gp350/220

Peptides with the CR2 binding site sequence on C3d and the viral coat protein gp350/220 of EBV inhibit B cell proliferation and EBV infection, respectively (Lernhardt *et*

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A

Complement C3	E	D	P	G	K	Q	L	Y	N	V	E	A	T	S
EBV coat protein	E	D	P	G			I	I	E	V	E	I	P	E
Interferon alpha	F	T	E	L	Q	Q	L	N	D	L	E	A	C	V

B

consensus sequence	91 100 QQLNDLEACV ++ +
IFNα1, IFNα13	.....
LeIF D	.....
IFNα2, (λa2)	.....
LeIF A	.....
IFNα6 (LeIF K)	.....
IFNα5 (LeIF G)	.....M
GX-1	.....M
IFNα14 (λ2h)	..M.....
LeIF H	..M.....
LeIF H1	..M.....
IFNα8	.....S..
LeIF B	VLC
IFNα16	.....
LeIF F	.....M....
IFN (Ovch)	.....
IFNα4b	.....
IFNα4a	.....
μ2c1 (LeIF I)	....N.....
IFNα88	....N.....
IFNα7 (LeIF J)	.....
IFLrK	.....
LeIF C	.....
$IFN\alpha_{II}1$	...QH..T.L
$IFN\alpha_{II}$ (P9A2)	...QH..T.L
$IFN\alpha_{II}$ (E76E9)	...QH..T.L

Fig. 1. Complement receptor type two (CR2) binding motifs. (**A**) Motifs on C3d, EBV coat protein and IFNα. (**B**) Motifs on IFNα subtypes. Shown are the amino acid sequences of complement component C3 (residues 1227–1232, underlined)and of Epstein Barr virus coat protein gp350/220 (residues 21–30) that form the complement receptor type two (CR2) binding sites (Lambris *et al.*, 1985; Nemerow *et al.*, 1989). Residues 87–100 of IFNα contain a putative CR2 binding site composed of residues 92–99. The amino acids coinciding with the consensus sequence are represented by dots. The positions conserved in all subtypes of human IFNs (Weissmann and Weber, 1986) are represented by a + sign.

al., 1987; Nemerow *et al.*, 1989). To test whether pIFNα may have the same anti-proliferative effects, we cultured Raji cells in the presence of increasing amounts of peptides and measured the incorporation of [^{3}H]thymidine into DNA. Figure 3A shows that, while the fibronectin-derived peptide (pFib) had no effect, pC3d and pIFNα inhibit Raji cell proliferation efficiently. EBV coat protein gp350/220-derived peptides with the CR2 binding motif have been shown to inhibit the infection of peripheral blood lymphocytes by EBV (Nemerow *et al.*, 1989). To test whether pIFNα inhibits the proliferation of EBV-infected B cells, we incubated peripheral blood B lymphocytes with EBV and peptides. Proliferation induced as a result of EBV infection was measured by [^{3}H]thymidine incorporation. Figure 3B shows that EBV-induced proliferation is greatly reduced by pIFNα but not affected by pSCR (a peptide with the scrambled sequence of the CR2 binding motif on C3d). Thus, the 92–99 motif peptide (pIFNα) displays the same activity as other peptides carrying CR2 binding motifs.

IFNα inhibits C3d/CR2 interaction

The ability of IFNα to inhibit the C3d/CR2 interaction on the cell membrane was assessed using particle-bound C3bi/C3d. Zymosan particles were coated covalently with complement fragment C3bi/C3d. Such particles bind CR2 in the membrane of Raji cells. This interaction can be observed in the light microscope as rosette formation which

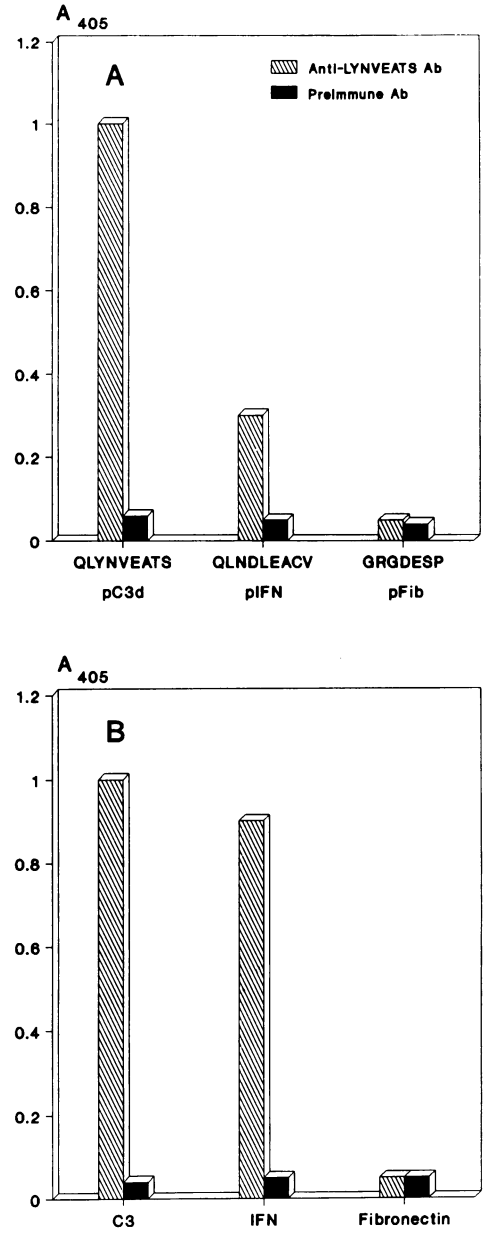

Fig. 2. Binding of anti-pC3d peptide antibodies to IFNα. (**A**) Binding of IgG fractions of polyclonal anti-pC3d antibodies (hatched bars) or of IgG from a preimmune serum (solid bars) to synthetic peptides derived from C3d, IFNα and fibronectin. (**B**) Binding of the same IgG fractions to the corresponding proteins. Synthetic peptides (10 μg), C3bi/C3d (200 ng), IFNαA (1 μg) and fibronectin (1 μg) were coupled to microtitration plates as described in Materials and methods. Anti-pC3d antibodies (dilution 1:1000) were then added to the wells followed by goat anti-rabbit alkaline phosphatase conjugated antibodies and chromogenic substrate. Positive signals were observed with anti-pC3d antibody dilution up to 1:10 000 (data not shown). The range between duplicates was typically less than 7%.

can be inhibited specifically with CR2 ligands such as soluble C3bi/C3d or anti-CR2 antibodies (Frade *et al.*, 1984) (Figure 4B). Figure 4A shows that pC3d and pIFNα inhibit rosette formation by 70% and 45% respectively, whereas pTN (a tenascin-derived peptide) has no effect. It appears that the C3d and IFNα peptides can assume conformations that compete with the binding of C3bi/C3d to native, membrane-associated CR2. Figure 4B shows that the recombinant C3 α chain (which includes the C3d fragment and hence the

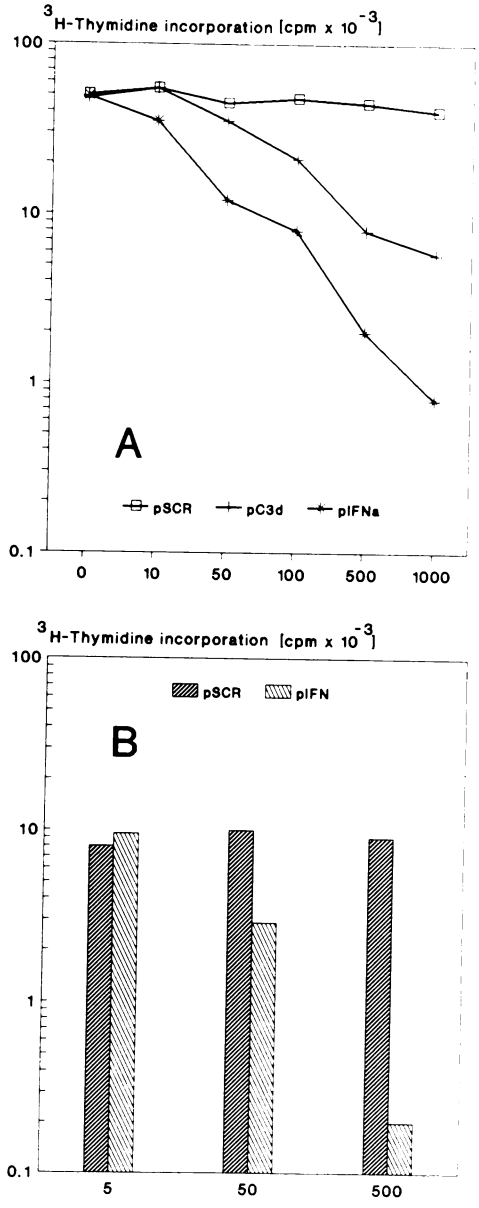

Fig. 3. Inhibition of B lymphocyte proliferation by pIFNα. (**A**) Inhibition of Raji cell proliferation. Raji cells (10^6/ml) were incubated with various amount of pC3d, pIFNα or pSCR (a peptide with the scrambled CR2 binding motif of C3d: YTLEAQVSN). Cell proliferation was measured after 48 h by a 4 h [^{3}H]thymidine pulse. (**B**) Inhibition of EBV-induced proliferation of peripheral blood B lymphocytes. Purified B cells (10^5/well) were infected with cell-free EBV supernatant from the B95-8 cells, a productively infected B cell line. EBV-induced B cell transformation was measured on day 6 by a 4 h [^{3}H]thymidine pulse.

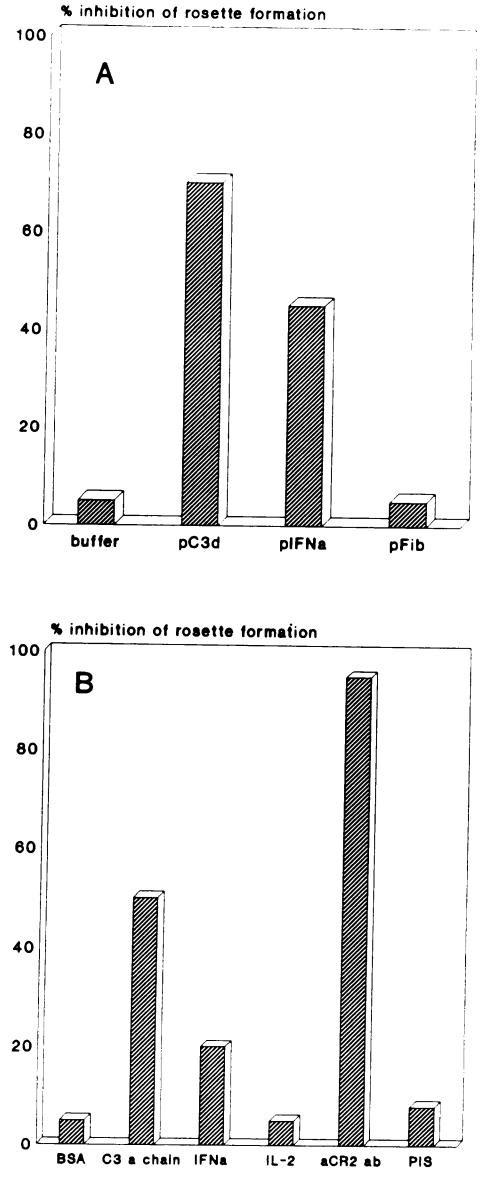

Fig. 4. Inhibition of Zymosan-C3bi/C3d rosette formation by IFNα. (**A**) Inhibition by synthetic peptides derived from C3 and IFNα. (**B**) Inhibition by recombinant C3α chain, eukaryotic IFNα A and anti-CR2 IgG (aCR2 ab). BSA, IL-2, IgG from preimmune serum (PIS) and peptides derived from fibronectin (pFib) were used as negative controls. Dose-dependent rosette inhibition was observed and the maximum inhibition was obtained with 10 μg peptide, 100 ng protein and IgG diluted to 1:2000 is shown. The values given represent the means of three different experiments carried out in duplicates.

CR2 binding site) inhibits rosette formation by 50%. Lower but specific inhibition (20%) is observed with recombinant IFNαA (rIFNαA) produced by a eukaryotic expression system. Identical results were obtained with prokaryotic rIFNαD (data not shown). Bovine serum albumin (BSA) and recombinant Interleukin-2 have no activity in this assay. These data show that IFNαA competes with particulate C3bi/C3d binding to native CR2 in the Raji cell membrane. Because the peptides pC3d and pIFNα show identical effects, our data suggest the presence of a functional CR2 binding site on IFNαA, and tentatively identify its location to the 92–99 motif.

IFNαA binds to CR2

To determine the direct interaction of IFNα with CR2 we devised a solid phase ELISA type binding assay. Increasing amounts of IFNα are used to coat the wells of microtiter plates and then a constant amount of purified CR2 is added. Bound CR2 is detected with the monoclonal anti-CR2 antibody HB5 (Nemerow *et al.*, 1989). For this set of experiments we chose to use rIFNαA produced by a eukaryotic expression system (eu.rIFNα) because, in our experience, eu.rIFNα has a higher specific activity than rIFNα produced by prokaryotic expression (pro.rIFNα), and pro.rIFNα–receptor complexes have been reported to be unstable (Faltynek *et al.*, 1983; Traub *et al.*, 1984). Figure 5 shows that CR2 binds IFNα in a dose-dependent way. In

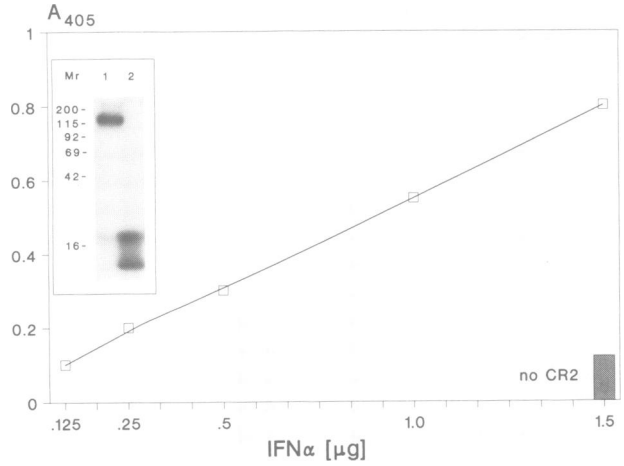

Fig. 5. Complex formation between IFNα and solubilized CR2. Varying amounts of IFNα were coated onto 96-wells plates and incubated with purified CR2. The monoclonal anti-CR2 antibody, HB5 (20 ng) and rabbit anti-mouse IgG alkaline phosphatase conjugate (dilution 1:1,000) were used to detect bound CR2. BSA, used as a blocking protein, served as negative control in the absence of CR2. Standard deviation between duplicates was usually less than 7%. **Inset**: aliquots of purified CR2 (lane 1) and eu.rIFNαA (lane 2) used in the ELISA were ^{125}I-radiolabelled and analyzed on a 10% SDS−PAGE.

this assay, BSA is used as an internal negative control since the microtiter wells are saturated with BSA prior to incubation with CR2. These data demonstrate that IFNαA, purified to homogeneity, can interact directly with purified CR2. Lower, yet specific, signals were obtained when using different subtypes of pro.rIFNα (IFNα2b, IFNαD, data not shown).

Because the anti-pC3d antibodies bind the 92−99 motif, these antibodies should disrupt the IFNα−CR2 interaction. Figure 6A shows that the anti-pC3d antibodies indeed inhibit this interaction suggesting that IFNα binding to CR2 may be mediated through the 92−99 sequence motif. IgG fractions directed against CR2 and IFNα (as positive controls) inhibit IFNα binding whereas preimmune IgG has no effect. Conversely, specific CR2 ligands such as C3bi/C3d and EBV coat protein gp350/220, but not BSA, inhibit IFNαA binding to CR2 (Figure 6B). Finally, IFNβ, which competes with IFNα for its receptor in cell binding assays (Merlin *et al.*, 1985), inhibits IFNαA interaction with CR2 (Figure 6B). IFNγ, known to bind a different receptor (Aguet *et al.*, 1988), has no effect. These data show that IFNα interacts with solubilized CR2 and that CR2 may be the cellular receptor that is shared by IFNα and IFNβ.

To confirm that CR2 is a receptor for IFNα, CR2 ligands, anti-CR2 and anti-IFNα antibodies were used to inhibit [^{125}I]IFNα binding to Raji cells. Binding constants were calculated from Scatchard plots and show that these cells express ~2000−5000 IFNα binding sites per cell with an apparent K_d of 10^{-9} to 10^{-10} M. These values are consistent with those previously described by others (Mogensen *et al.*, 1981; Langer and Pestka, 1986). As shown in Figure 7A, anti-CR2 and anti-IFNα IgG inhibit the binding of [^{125}I]IFNα to Raji cells in a dose-dependent manner whereas preimmune IgG has no effect. Figure 7B shows that C3bi/C3d partially inhibits binding, while a transferrin control shows no reduction in bound [^{125}I]IFNα. To confirm the role of the CR2 binding motif on IFNα, experiments were carried out in the presence of increasing amounts of pIFNα and pC3d. Figure 7C shows that monomeric or polymeric forms of pC3d (coupled to BSA)

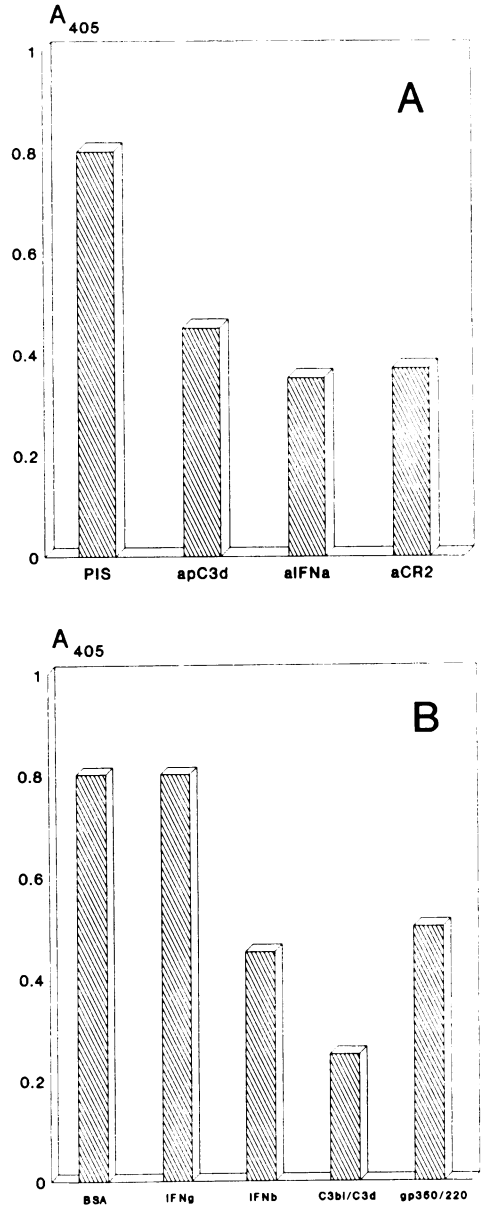

Fig. 6. Inhibition of IFNα binding to solubilized CR2. (**A**) Binding of IFNα to CR2 in the presence of IgG fraction of polyclonal anti-pC3d (apC3d), anti-IFNα (aIFNα), anti-CR2 antibodies (aCR2) and of preimmune serum (PIS). (**B**) Binding experiments in the presence of CR2 ligands and different types of rIFN. ELISAs were carried out as described in Figure 5. Data presented were obtained with antibodies diluted to 1:1000 and in the presence of 200 ng C3bi/C3d or recombinant gp350/220 and 50 ng rIFN and rIFNγ.

inhibit [^{125}I]IFNα binding to Raji cells. Remarkably, the binding is nearly completely abolished. Binding of [^{125}I]IFNα to Raji cells is not affected by monomeric or polymeric pTN. The IFNα-derived peptide inhibits this binding reaction to 75%. Higher doses of pIFNα show less inhibition possibly due to aggregation of this peptide at high concentrations. These results confirm that CR2 is a cellular site for IFNα binding to Raji cells, and that this interaction is mediated by the CR2 binding motif.

Additional inhibition experiments were carried out in the presence of monoclonal anti-CR2 antibodies HB5 (Tedder *et al.*, 1984) and OKB7 (Mittler *et al.*, 1983). CR2 is made up of 15−16 small consensus repeats (SCR) (Moore *et al.*, 1987; Weis *et al.*, 1988). HB5 recognizes an epitope located

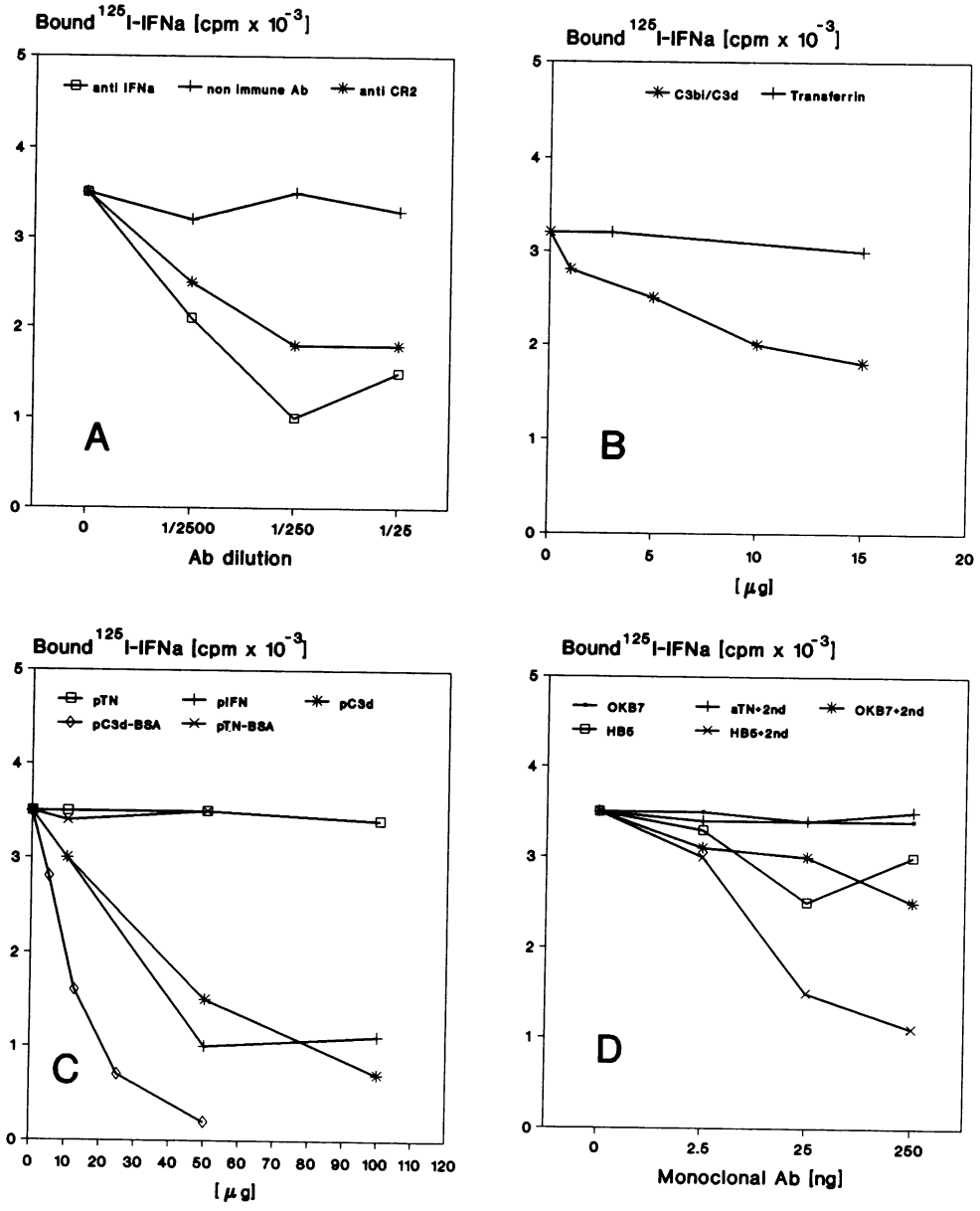

Fig. 7. Inhibition of [^{125}I]IFNα binding to Raji cells. (**A**) Inhibition by polyclonal antibodies. (**B**) Inhibition by C3bi/C3d. (**C**) Inhibition by monomeric synthetic peptides derived from C3d, IFNα and Tenascin (pTN) and by polymeric peptides (conjugated to BSA) derived from C3d (pC3d-BSA) and Tenascin (pTN-BSA). (**D**) Inhibition by monoclonal anti-CR2 antibodies alone (HB5, OKB-7) and in the presence of rabbit anti-mouse IgG (HB5+2nd, OKB-7+2nd). Monoclonal anti-Tenascin antibody in the absence or presence of secondary antibody (aTN±2nd) was used as negative control. Binding experiments were carried out on Raji cells (10^6) and an amount of [^{125}I]IFNα2b to yield 50% of binding site occupation as previously determined by titration. Each assay was carried out in triplicate (standard deviation was less than 8%) and the background (determined in the presence of 100-fold excess cold IFNα) was subtracted.

in a region composed of SCR-3, SCR-4 and part of SCR-5, while OKB7 recognizes a region in close proximity to the binding site for C3d and EBV, i.e. SCR-1 and SCR-2 (Lowell *et al.*, 1989; Carel *et al.*, 1990). OKB7 inhibits C3d and EBV binding to membrane associated CR2 whereas HB5 inhibits binding of these ligands only in the presence of secondary antibodies such as rabbit anti-mouse IgG (Ahearn and Fearon, 1989). It should be noted, that in contrast to the cell binding assays presented here, in solid phase assays with solubilized CR2 such as described above, neither OKB7 (Myones and Ross, 1987) nor HB5 (Nemerow *et al.*, 1989) (Figures 5 and 6) inhibit ligand binding, not even in the presence of secondary antibodies. Figure 7D shows that HB5 alone slightly inhibits [^{125}I]IFNα binding to Raji cells whereas in the presence of secondary antibodies it inhibits to 65%. Surprisingly, OKB7 alone has no inhibitory effect. In the presence of secondary antibodies, low inhibition (30%) is observed. As a control, secondary antibodies, alone or in the presence of anti-tenascin monoclonal antibody, have no inhibitory effect. These data suggest that the IFNα receptor on Raji cells carries the HB5 epitope, hence providing further evidence that CR2 molecules serve as receptors for IFNα on Raji cells.

Discussion

In the present study, we describe the interaction of IFNα with complement receptor type 2. Using synthetic peptides, we accumulated evidence that IFNα binding to B cells was mediated through a CR2 binding motif and, by using

monoclonal antibodies of known specificities, identified CR2 as a cellular receptor involved in IFNα/B cell interaction.

The direct interaction between IFNα and CR2 was established in three series of experiments. First, we found that IFNαA partially but specifically inhibited particulate C3bi/C3d interaction with CR2 on Raji cells. Second, using a solid phase binding assay, we showed that purified recombinant IFNα interacted with solubilized CR2 in a dose-dependent manner. This interaction was detected using rIFNαA produced by eukaryotic expression. Different IFNα subtypes produced by prokaryotic expression (pro.rIFNαD, IFNα2b) interacted with CR2 to a lesser extent (data not shown). IFNα binding to solubilized CR2 was inhibited by polyclonal anti-CR2 and anti-IFNα antibodies. In addition, C3bi/C3d, gp350/220 and IFNβ were also found to inhibit IFNα binding. Third, we demonstrated that binding of [^{125}I]IFNα to Raji cells could be specifically inhibited by polyclonal anti-CR2 and monoclonal anti-CR2 antibody (HB5) and also partially by C3bi/C3d. We chose the Burkitt lymphoma Raji for these studies because, in this cell line, down-regulation of IFNα receptors observed upon binding of its ligand is low (Hannigan *et al.*, 1984; Pfeffer and Donner, 1990) and because internalization of CR2 is unlikely (Tedder *et al.*, 1986). Our findings also show that the IFNα/CR2 interaction is probably mediated through the C3d/EBV binding region on CR2 molecules.

The involvement of IFNα residues 92–99 in IFNα/CR2 interaction was addressed in several experiments. First, polyclonal antibodies raised against a peptide carrying the CR2 binding site on C3d revealed that the IFNαA peptide (QLNDLEACV) and pC3d (QLYNVEATS) express common epitope(s). We also found that anti-pC3d antibodies bound to both C3bi/C3d and IFNα molecules, and inhibited IFNα binding to solubilized CR2. Second, we demonstrated that pIFNα mimics C3d and EBV gp350/220 derived peptides in that it inhibits Raji cell proliferation and the proliferation of EBV-infected B cells. Third, we showed that pC3d and pIFNα specifically inhibited the interaction of particle-bound C3bi/C3d and [^{125}I]IFNα with CR2 on Raji cells. These data strongly suggest that IFNαA residues 92–99 constitute a functional CR2 binding motif that mediates IFNα binding to CR2 on B cells. The contribution of individual amino acids within this binding site remains to be elucidated. Preliminary results, obtained with synthetic peptides bearing the 92–99 motif from subtypes LEIF-H (QMNDLEACV) and IFNα88 (QLNNLEACV) (see Figure 1B), suggest that the substitution of leucine with methionine at position 93 abrogates CR2 binding whereas the substitution of aspartic acid by asparagine at position 95 has no effect. Although precursory, these findings may indicate that some subsets of IFNα will not bind CR2. Substitution of other amino acids within the 92–99 motif by site directed mutagenesis will reveal the relative contribution of each to CR2 binding.

Because about 80% of IFNα binding to Raji cells was inhibited by pC3d, and given the proven specificity of monoclonal antibody HB5 used in our assays, our data indicate that CR2 may indeed be the main IFNα receptor on B lymphocytes. The surprising observation that, unlike other CR2 ligands, monoclonal antibody OKB-7 did not significantly inhibit IFNα binding to Raji cells, raises the question of whether IFNα binds CR2 proper or a CR2 subpopulation lacking the OKB-7 epitope. The latter may explain the discrepancy between the number of CR2 molecules on B cell lines (20 000–70 000) (Ahearn and Fearon, 1989) and the number of high affinity IFNα-binding sites found by us and others (2000 to 5000) (Langer and Pestka, 1986). In such a scenario IFNα receptors would represent an OKB-7 minus subpopulation amounting to 7-10% of CR2 molecules. CR2 variants are known to exist because differential expression of CR2 epitopes (such as determinants recognized by monoclonal antibodies HB5, OKB-7 and AB2) has been described for some subsets of B cells and on other cell types (Cooper *et al.*, 1988; Young *et al.*, 1989; Sauvageau *et al.*, 1990). For instance, epithelial cells could be stained with HB5 but not with OKB-7 (Young *et al.*, 1989). Other studies suggest that certain cells may express only an 'EBV receptor' while others express only a 'C3d receptor' (Cooper *et al.*, 1988; Ahearn and Fearon, 1989). Such findings appear to contradict the concept of C3d and EBV binding to the same receptor molecule. However, this discrepancy may be explained by specific interaction of these different ligands with subsets of CR2. This possibility is supported by three findings. First, although similar, the CR2 binding motif of C3d, gp350/220 and IFNα are not identical; this could account for their different binding properties. Indeed, C3d and gp350/220 carry an EDPG sequence (Figure 1A) that is not found on IFNα. The EDPG sequence has been shown to be crucial for the binding of gp350/220-derived peptides to CR2 (Nemerow *et al.*, 1989) and its absence in IFNα could explain why CR2 ligands only partially inhibited IFNα binding to CR2. Second, C3d and gp350/220 may interact with distinct sites on CR2 (Barel *et al.*, 1988). Third, only one ligand binding site has been detected on CR2 (Lowell *et al.*, 1989; Moore *et al.*, 1989; Carel *et al.*, 1990). Thus, although peptides bearing CR2 binding motifs appear not to display subset specificity, it is possible that the corresponding proteins interact with different CR2 subsets rather than with different sites on the same molecule. CR2 or related molecules could constitute a family of receptors from which subsets would interact specifically with different CR2 ligands. The genetic composition of the CR2 gene provides for the possibility of differential exon usage which may be a pathway for the generation of CR2 subtypes (Fujisaku *et al.*, 1989; Toothaker *et al.*, 1989; Holguin *et al.*, 1990). Two subtypes, distinguished by an additional SCR that is inserted between SCRs ten and eleven are known to be coexpressed in Raji cells and tonsillar B lymphocytes (Toothaker *et al.*, 1989). In addition, the CR2 gene structure revealed three 'CR1-like' exons, located between the leader signal exon and the SCR1 and 2 encoding exon (Holguin *et al.*, 1990). By homology to the murine CR2 gene (Kurtz *et al.*, 1990), these exons could encode two SCRs each. Incorporation of alternative SCRs by differential splicing pathways may be the mechanism of CR2 subset generation.

CR2 molecules are likely receptor candidates for IFNα because both molecules affect B cell proliferation (Ahearn and Fearon, 1989; Pestka *et al.*, 1987). An interesting feature of CR2 is the capacity of this receptor to trigger stimulatory or inhibitory effects. It appears that these events are ligand-valency dependent. Polymeric C3d has been shown to enhance, whereas monomeric C3d has been found to inhibit B cell proliferation (Melchers *et al.*, 1985). Studies with peptides also support the ligand-valency notion. Peptides with the CR2 binding motif inhibit B cell proliferation, whereas

crosslinked CR2 binding peptides have been shown to stimulate B cell proliferation (Lernhardt *et al*, 1987; Servis and Lambris, 1988). In this context, it is conceivable that CR2 could mediate IFNα's known anti-proliferative effect. Whether chemically crosslinked IFNα has stimulatory effects is under investigation. CR2 is also an ideal target for IFNα's anti-viral activity since this receptor is the necessary membrane site for cellular attachment of EBV, a prerequisite for B cell infection (Fingeroth *et al.*, 1984). Previous studies have shown that preincubation of B cells with IFNα prevents EBV infection (Lotz *et al.*, 1985) and we showed here that pIFNα abrogates EBV-induced B cell proliferation. The mechanism of this inhibition of B cell infection is not known yet. The exact role of CR2 as a mediator of anti-proliferative and/or anti-viral activities of IFNα needs further clarification.

Previous studies on IFNα receptor characterization were mainly oriented towards the identification of a gene product encoded by chromosome 21 because the ability to bind IFNα has been mapped to this chromosome (Stewart, 1979). Recently, a cDNA coding for such a receptor has been cloned (Uze *et al.*, 1990). However, the authors noted that the protein encoded by the cDNA was not by itself sufficient for the complete binding and transduction of IFNα activities. Also, the ligand-specificity of the protein was restricted to the IFNαB subtype and the sequence motif on IFNαB involved in this interaction is not known. The predicted amino acid sequence from the cloned receptor differs from the amino acid sequence of CR2, which is encoded by chromosome 1 (Moore *et al.*, 1987). Thus, while the presence of another receptor for IFNαB has been shown, CR2 or a subpopulation thereof may represent a common receptor for some IFNα subtypes and for IFNβ on B cells. The identification of the sequence QLNDLEACV and other sequences involved in IFNα binding to B cells may open new approaches to IFNα therapy of B cell malignancies and possibly of EBV infection.

Materials and methods

Materials

Eukaryotic rIFNαA, rC3α chain and rIL2 were obtained from L cells transfected with the eukaryotic expression vector BCMGShyg (Karasuyama *et al.*, 1989), containing IFNαA cDNA (LEIF-A, gift from P.Gray), C3α chain cDNA (gift from G.Fey), and IL-2 cDNA (gift from H.Karasuyama). The expression of C3 α chain and rIFNαA was verified in ELISAs using monoclonal anti-C3d Ab (Quidel, San Diego, CA), and polyclonal anti-IFNα (Boehringer Mannheim, Indianapolis, IN). IL-2 activity was assessed in the HT-2 proliferation assay. Prokaryotic rIFNαD was a gift from Hoffmann La Roche, Nutley, NJ. Prokaryotic rIFNα IFNγ and IFNβ were a gift from Amgen, Thousand Oaks CA. Prokaryotic IFNα2b and synthetic peptides were purchased from Schering, Kenilworth, NJ and Multiple System Peptides, La Jolla, CA, respectively. C3 was purchased from Calbiochem, La Jolla, CA and C3bi/C3d was obtained by controlled proteolysis of C3. Gp350/220 was a gift from G.Nemerow.

Antibodies

Polyclonal anti-pC3d antibodies were raised by immunizing rabbits with pC3d covalently linked to BSA. Briefly, 1 mg pC3d was incubated with 1 mg BSA in 0.1 M phosphate buffer pH 6.8, 0.25% glutaraldehyde for 3 h at 22°C. After blocking in 1 M Tris−HCl pH 7 for 2 h and extensive dialysis in PBS, three aliquots were injected weekly. Specific anti-pC3d antibodies were detected by ELISA. Once a positive antiserum was obtained, the IgG fraction of this serum was preadsorbed several times on Sepharose−BSA to minimize its anti-BSA titer. Polyclonal anti-IFNα and anti-CR2 antisera were prepared by immunizing rabbits with a polyacrylamide gel suspension obtained from an SDS−PAGE slice containing the purified corresponding proteins in complete Freund's adjuvant.

IFNα and CR2 purification

Recombinant eukaryotic rIFNαA purification: L cells transfected with BCMGSVhyg-LEIF-A were cultured in serum-free MEM Iscove's medium (Boehringer Mannheim) containing 100 μg/ml BSA and 18 μg/ml transferrin. Typically, 650 U/ml of IFNα were obtained as determined in a viral cytopathicity protection assay as described previously (Lotz *et al.*, 1985). Proteins from 3 l of supernatant were concentrated by 70% ammonium sulfate precipitation and resuspended in 300 mM phosphate buffer pH 6. After dialysis, high molecular weight proteins, aggregated and polymeric forms of IFNα were separated from monomeric IFNα by gel filtration on BioGel P60 (BioRad, Richmond, CA). IFNα elution was followed by ELISA using monoclonal and polyclonal horse anti-IFNα antibodies (Boehringer Mannheim). IFNα was then further purified by affinity chromatography using polyclonal horse anti-IFNα antibody covalently bound to Affigel-10 beads (BioRad). Purified IFNα was stored at −20°C in 300 mM phosphate buffer pH 6.

CR2 purification: Plasma membrane preparation from Raji cells (10^6 in 50 μl PBS) and protein extraction were carried out as previously described (Delcayre *et al.*, 1987). Solubilized CR2 was isolated by two successive affinity chromatographies using a mixture of monoclonal anti-CR2 antibodies, OKB-7 (OrthoImmune, Raritan, NJ) and HB5 (Becton-Dickinson, Rutherford, NJ). Bound proteins were eluted in 20 mM diethylamine pH 11.5, 150 mM NaCl, 0.1% Triton X-100. Purified CR2 was stored at −80°C in PBS 0.1% Triton X-100.

Inhibition of particulate C3bi/C3d-binding to Raji cells

Particulate C3bi/C3d was prepared by incubation of fresh human serum with Zymosan A beads (Sigma, St. Louis, MO) for 30 min at 37°C (Frade *et al.*, 1984). Raji cells (10^6 in 50 μl PBS) were preincubated for 20 min at 37°C with different CR2 ligands as indicated in Figure 4 prior to incubation with C3bi/C3d coated Zymosan. After 10 min incubation at 37°C cells were centrifuged and incubation was continued for 45 min at 37°C. Rosette formation was observed under the microscope and cells bearing three beads or more were counted positive.

ELISA for detecting CR2 binding motif

Synthetic peptides (10 μg), C3bi/C3d (200 ng), rIFNαA (1 μg), and 1 μg fibronectin (gift from M.Bourdon) in 100 μl of 0.1 M sodium bicarbonate buffer pH 9.6 were coupled overnight at room temperature to microtitration plates (Linbro, Flow Labs, McLean, VA). After washing three times in PBS wells were saturated by addition of 200 μl Tween 20 (0.05% in PBS) for 30 min at room temperature. Coupled peptides and proteins were incubated for 1 h at 22°C with 100 μl of IgG fractions of polyclonal anti-pC3d antibodies or of preimmune serum (dilution 1:1000 in PBS, 0.05% Tween 20, 0.1% BSA). The wells were washed three times in the same buffer and then incubated for 45 min at 22°C with 100 μl goat anti-rabbit IgG coupled to alkaline phosphatase (dilution 1:1000; Jackson Immuno Research Labs, West Grove, PA). After several washes, the wells were finally incubated with 100 μl of 1 mg/ml chromogenic substrate (Sigma 104 Phosphatase Substrate, Sigma, St. Louis, MO) in 10 mM Tris−HCl pH 8.5, 150 mM NaCl and the absorbance at 405 nm was determined in a Titertek II ELISA reader (Flow Labs, McLean, VA).

Cell proliferation assays

Raji cells were cultured in Iscove's serum free medium at 2×10^5/200 μl in microtitration plates in the presence of varying amounts of peptides as indicated in Figure 3. Proliferation was assessed after 48 h by a 4 h pulse with [^{3}H]thymidine (New England Nuclear, Boston MA; 740 GBq/mM, 1 μCi per well). Peripheral blood B lymphocytes were purified and infected with EBV using supernatants from productively infected B95-8 cells as described by Lotz *et al.*, 1985. Peptides were added at initiation of culture at the concentration indicated. EBV-induced proliferation was measured by a 4 h [^{3}H]-thymidine incorporation on day 6.

Solid phase binding assay

Increasing amounts of purified eu.rIFNαA were coated to microtiter plates as described for ELISA above. After blocking with Tween 20 and BSA, solubilized CR2 (100 ng in 100 μl) was added to the wells and incubated for 2 h at 37°C. Wells were washed three times in PBS, 0.05% Tween 20, 0.1% BSA and incubated with 100 μl of 1:50 dilution of HB5 in the same buffer for 1 h at 22°C, then with rabbit polyclonal anti-mouse IgG conjugated to alkaline phosphatase (dilution 1:1000, 100 μl). Absorbance at 405 nm was determined as mentioned above. In inhibition experiments, CR2 was added in the presence of different inhibitors, as described in the legend to Figure 6.

Binding experiments with [^{125}I]IFNα2b

Prokaryotic rIFNα2b was ^{125}I-radiolabelled using the iodogen technique (Markwell and Fox, 1978). Binding experiments of [^{125}I]IFNα (5×10^6 c.p.m./μg) to Raji cells were carried out following previously described procedures (Mogensen *et al.*, 1981; Langer and Pestka, 1986). Briefly, 10^6 Raji cells in 50 μl PBS, 0.2% BSA were incubated with iodinated IFNα (5×10^5 c.p.m.) and with the different ligands indicated in Figure 7. After 1 h incubation at 4°C, unbound material was removed by centrifugation over a layer of mineral oil. The cell pellets containing bound IFNα were counted in a gamma counter.

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