Cutting Edge: Novel RNA Ligands Able to Bind CD4 Antigen and Inhibit CD4+ T Lymphocyte Function

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The value of high affinity-specific reagents in immunology is exemplified by the use of mAbs. Recent in vitro selection methods suggested that oligonucleotides may provide a useful alternative, especially where Abs have been insufficient thus far. We used a systematic evolution of ligands by exponential enrichment (SELEX) procedure to derive high affinity oligonucleotide ligands (aptamers) recognizing CD4. These RNAse-resistant aptamers bound with high affinity and specificity as demonstrated using BIAcore (Stevenage, U.K.) technology. They also bound native CD4 on rat lymphocytes and specifically interfered with labeling by high affinity mAbs. All aptamers recognized the same binding site in the CDR2-like region in domain 1 of CD4. The applicability of these aptamers for immunologic studies was clearly demonstrated by their ability to block a fully allogeneic MLR in a CD4-specific manner. The high affinity and stability of aptamers point to their value in the analysis and functional manipulation of the immune system. The Journal of Immunology, 1998, 160: 5209–5212.

The study of immunology was revolutionized when the mAb technique became widely applied. The advantages of mAbs were that they were highly specific, were stable, could be produced in large amounts, and were of high affinity. For these reasons, they were widely used to block interactions, to purify or localize target proteins, and to act as surrogate ligands in cellular reactions. Because mAbs are traditionally raised in laboratory animals, one clear disadvantage is that their production is limited to Ags that are immunogenic in the systems used, although some progress has been made in bypassing this by the in vitro production of Abs (1).

The systematic evolution of ligands by exponential enrichment (SELEX) method is based on the in vitro selection of rare oligonucleotide ligands followed by their amplification and cloning. The so-called “aptamers” that are isolated by this method display a high affinity and specificity to the target molecule on which they are selected (2). The limited biochemical diversity of nucleotides compared with amino acids in proteins is offset by the large complexities of libraries of potential aptamers that can be easily produced and investigated. The SELEX method involves synthesizing DNA oligonucleotides that contain a region of random sequence flanked by constant regions that allow amplification and transcription. A typical library has a complexity of about $10^{15}$, which is then transcribed into RNA. These RNA molecules adopt a multiplicity of structures, which allows some of them to bind to a given target structure. RNA that binds to the target is separated from unbound, and, utilizing the fixed regions, the separated RNA is reverse transcribed and PCR-amplified (3, 4) to produce a template for the next round of selection. This cycle is repeated until individual clones and hence aptamers can be obtained (2).

To use such aptamers for diagnostic or therapeutic uses, they need to be resistant to nucleases that are commonly found in body fluids. It has been demonstrated that RNA molecules containing a fluoro (F) or an amino (NH$_2$) group at the 2’ position of the ribose of pyrimidines are sufficiently RNAse resistant to allow such applications (5–8). These modifications are not only compatible with the enzymatic processes involved in the SELEX process (transcription and reverse transcription), they also widen the spectrum of potential tertiary structures due to their different chemistry. In addition, especially fluoro-substituted RNAs give rise to more compact and rigid aptamers with a likelihood of higher affinity compared with standard RNA or amino-modified RNA aptamers (9).

The CD4 Ag, which contains four Ig-like domains in the extracellular region, is an accessory protein in Ag recognition via its interaction with MHC Class II on APCs. It is also a receptor for HIV-1 (10, 11). Abs recognizing CD4 can block both Ag presentation and HIV binding (12–14). Abs against CD4 are also commonly used to induce tolerance in animal model systems of human diseases (15). We describe the production of RNA-aptamers against rat CD4 using 2’-modified nucleotides and recombinant CD4. Their specificity and value as tools were evaluated by surface plasmon resonance in the BIAcore (Stevenage, U.K.), flow cytometry, and in functional analysis in mixed lymphocyte reactions.

Materials and Methods

The SELEX process

Technical details of the SELEX process were largely as described elsewhere (16). The random region consisted of 36 residues flanked by 24 and 23 residues on the 5’ and 3’ side, respectively, giving a full sequence of 83. The oligonucleotide library was produced by Genosys (Cambridge, U.K.).
Two modified pyrimidine nucleotides (Amerham, U.K.), together with unmodified purine nucleotides, were used for in vitro transcriptions with an optimized transcription buffer (17). Recombinant sCD4 was immobilized on Sepharose (Pharmacia, Milton Keynes, U.K.). Purification was performed in modified PBS (1 mM MgCl₂, 1 mM CaCl₂) with the Sepharose-immobilized sCD4 for 24 h in the first round and for 2.5 h in all the following rounds. After the incubation, beads were washed five times in binding buffer, and bound RNA was eluted with 7 M urea followed by phenol/chloroform treatment. The recovered RNA was reverse transcribed with avian myeloblastosis virus (AMV) reverse transcriptase (Pharmacia), and the product was amplified via PCR under slightly mutagenic conditions (16) to give rise to a new template for the next round of selection. To reduce inadvertent selection for RNAs that bind to the Sepharose matrix, absorption steps using uncoupled Sepharose 4B beads were incorporated every third round. After 15 rounds of selection, the resulting cDNA was amplified with primers 1 and 2: CCGAAGCTTAATACGACTCACTATAGGGAGACAAGAGCTGCATACTTGAG (primer 1: GCCGGATCCGCCTGTTGTGAGCCTCCTGTCGAA; and primer 2: CCGAAGCTTTATAGCCTCTATATGGAGACAGAATACACGTCCAA; and primer 2: ATCGTACAGCTGAA) (15). The PCR product was digested with both nucleases and cloned into pUC19 vector. Single clones were isolated and sequenced using an Applied Biosystems 360 automatic sequencer (Warrington, U.K.).

**Blacore analysis**

For Blacore analysis, 25 µl of RNA, at a concentration of about 100 µg/ml in modified HBSS (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, and 1 mM CaCl₂), was injected into five flow cells where rat sCD4 was immobilized directly onto the surface of the chip using an amine coupling kit and sCD4 at 35 µg/ml in 10 mM sodium acetate (pH 5.0). Results were evaluated with the Blacore evaluation III program (Blacore).

**Flow cytometry**

Rat lymph node cells (2 × 10⁵) were preincubated for 15 min with 1 µg RNA in 100 µl before addition of saturating amounts of Ab. After 30 min, cells were washed and stained with FITC-coupled rabbit anti-mouse Abs (Pierce, Chester, U.K.). Five thousand live-gated events were recorded in a FACSScan (Becton Dickinson, Oxford, U.K.) and analyzed with the CellQuest software. Abs MRC OX21 (directed against human C3b inactivator) (20); MRC OX8 (anti-CD8) and W3/25 (anti-CD4) (21); and MRC OX 63 and OX 65 (both anti-CD4) (14) were used as tissue culture supernatants.

**Mixed lymphocyte reactions and proliferation assays**

Lymph node responders (5 × 10⁴) of the PVM strain of rats were incubated with 5 × 10⁵ irradiated (3000 rad) splenic stimulator cells of the DA strain in 96-well plates for 3 days, followed by a pulse with [³H]thymidine for 18 h. The cells were harvested and analyzed in a β-plate reader (Wallac, Milton Keynes, U.K.). Where indicated, RNA, W3/25 IgG, or W3/25 Fab fragments were added to the responder cells 15 min before stimulator cells were added.

**Results and Discussion**

**In vitro selection of RNA aptamers that bind to recombinant rat CD4 immobilized on Sepharose.**

In an effort to identify high affinity RNA ligands for rat CD4, a pool of about 5 × 10¹⁴ different RNA molecules was transcribed from a DNA library randomized at 36 positions. This RNA was subjected to 15 rounds of an in vitro selection procedure with recombinant rat sCD4 as the target. Using the Blacore, weak signals could be seen after as few as six rounds (data not shown) and strong specific binding to rat sCD4 after 10 rounds of selection. After 15 rounds of selection, clones of RNA aptamers were isolated and individually tested for specific binding to rat sCD4. Specificity of binding was demonstrated for two selected aptamers by testing them also on human sCD4, which has approximately 53% sequence identity to rat CD4 (Fig. 1, A and C).

**RNA sequences of CD4-specific aptamers**

The PCR product after the last round of selection was cloned into pUC19, with 32 clones being analyzed separately for CD4 binding. Ten clones (31% of total) showed specific binding to sCD4, and sequences of these were grouped according to sequence similarity into three different families and two orphan clones (Fig. 2). This result clearly demonstrates that there are several independent solutions for specific high affinity binding of RNA to rat sCD4, as demonstrated for other targets by others (9, 22, 23). Preliminary Blacore kinetic analysis suggested an affinity in the low nanomolar range.

**Mapping of the binding site of CD4-specific aptamers**

To map the binding site of the aptamers on CD4 (for which we would like to suggest the name “aptatope”), rat lymph node cells were preincubated with RNA from representative clones from each group of aptamers and then stained with CD4-specific mAbs or with mAbs against other cell surface molecules. Only staining with the high affinity mAbs MRC OX63 and W3/25 (24) was affected by aptamers, suggesting that the exposed CDR2-like loop recognized by these Abs (14) was the actual aptatope. The degree of blocking varied, with some clones reducing labeling by only 10%, whereas clone 14 gave 90% inhibition when OX63 staining was

**FIGURE 1.** Blacore analysis of aptamer specificity. A, RNA from unselected pools (injection 1), round 10 (injection 2), and round 11 (injection 3) was injected into flow cells where either 5000 response units (RU) of rat CD4 were immobilized (solid lines) or control protein (albumin, interrupted lines, 10,000 RU). Only selected RNA bound to rat CD4, and neither selected nor unselected RNA bound to the albumin control; B and C show the specific binding of two cloned aptamers (clones 8 and 14, respectively) to rat CD4 (solid lines) but not to human CD4 (interrupted lines).
investigated (Fig. 3). None of the CD4-specific aptamers gave significant blocking of Abs recognizing different regions of CD4 or other lymphocyte surface Ags (Fig. 3, and data not shown). Thus, it seems as if all the different RNA species isolated bind to the aptatope on CD4, irrespective of their different sequences. The existence of dominant aptatopes has been observed for other protein-aptamer interactions before (25). Why some sites make particularly good aptatopes is not yet known but may well have something to do with the local distribution of basic residues in particular regions of proteins.

RNA aptamers have the capacity to inhibit mixed lymphocyte reactions

The ability of aptamers to block functional assays was tested using a mixed lymphocyte reaction where the W3/25 CD4 mAb, which binds the same site as the tested aptamer clones (Fig. 3) and is of particularly high affinity (24), gave effective inhibition (26). Clear inhibition by CD4-specific aptamers by about 50% was observed in the fully allogeneic mixed lymphocyte reactions with an RNA concentration of 5 μg/ml, as illustrated for one experiment in Figure 4. No inhibitory effects due to nonspecific cytotoxicity of added RNA could be observed with a polyclonal RNA control. Clone 8, which binds CD4 less well and has a different sequence (Fig. 2), gave partial inhibition (Fig. 3, and data not shown). Thus the ability to block the MLR correlated with CD4 binding activity. The inhibition observed with the monovalent RNA is clearly less than with bivalent W3/25 IgG. Fab fragments of W3/25 were less effective than the full IgG and were similar to the aptamers in their ability to inhibit a MLR. It cannot be excluded that the activity of the Fab may be due to minor levels of contamination with intact IgG or to aggregation of the Fab.

Aptamers so far have been shown to be able to block a variety of interactions, including binding of cytokines to their receptors (22), the binding of CD62L or L-selectin (27), and (so far only for DNA aptamers) in short term in vivo systems (27, 28). Our study here shows that CD4-specific RNA aptamers could be readily produced and had sufficient affinity and lifespan to give effective inhibition of an interaction involving very strong stimuli in a fully allogeneic mixed lymphocyte interaction. It seems feasible that aptamers will be of value in the manipulation of the immune system. This might be especially true when highly conserved proteins are to be targeted, where there is a difficulty in raising high affinity mAbs because of tolerance across species barriers.

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