Thioaptamer decoy targeting of AP-1 proteins influences cytokine expression and the outcome of arenavirus infections

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INTRODUCTION

Members of the family Arenaviridae (genus Arenavirus) are small RNA viruses that include the prototype Lymphocytic choriomeningitis virus (LCMV), Pichinde virus (PICV) that is non-pathogenic for humans, and highly pathogenic viruses that cause viral haemorrhagic fevers (VHF), including Lassa fever and Argentine, Venezuelan and Bolivian haemorrhagic fevers. Although arenaviruses infect macrophages and dendritic cells, the immune response to arenavirus infections is poorly understood and, as with many of the viruses causing VHF, it is unclear how these viruses evade the immune system or cause death with only limited cellular damage. Our laboratory has been using PICV to study the effects on the immune response of both lethal and non-lethal arenavirus infection of guinea pigs. Increases in TNF-α have been associated with fatal PICV infection of guinea pigs (Aronson et al., 1995), but only in the late stages of infection. Alternatively, there is evidence that swift elaboration of pro-inflammatory cytokines and early engagement of the innate immune response may help protect an infected host from lethal haemorrhagic fever (Baize et al., 1999; Peters et al., 1987; Leroy et al., 2000; Fisher-Hoch et al., 1988). We have been interested in developing strategies to interfere with the pathogenic sequence, or even boost early protective innate immune responses.

Members of the AP-1 family of transcription factors are key regulators of a wide range of cellular processes including cell proliferation, cell death, cell differentiation, oncogenesis, inflammation and innate immune responses (Adcock, 1997). While not as strongly associated with the immune response as the NF-κB family of transcription factors, it is still involved in the transcriptional regulation of T-cell receptor alpha (Giese et al., 1995), beta-interferon (IFN-β) (Du et al., 1993; Merika et al., 1998; Thanos & Maniatis, 1985).
1995), TNF-α (Falvo et al., 2000; Tsai et al., 2000) and other genes important to an antiviral immune response (Chinenov & Kerppola, 2001).

Similar to the NF-κB family, the AP-1 transcription factors are composed of various dimers which can act as positive or negative regulators of transcription. The subunits are basic region–leucine zipper proteins of the Jun, Fos, Maf, ATF and cAMP response element-binding (CREB) protein subfamilies. The best-studied activator dimers are the Fos/Jun dimers, which bind to the canonical AP-1 site, and the CREB dimers, which bind to the similar CRE sites with some level of cross-binding. The significance of the various dimer combinations and binding sites are not fully understood at this time (Shaulian & Karin, 2001, 2002; Karin & Shaulian, 2002, 1998). The regulation of AP-1 activity is complex and occurs through: (i) changes in jun and fos transcription and mRNA turnover, (ii) Fos and Jun protein turnover, (iii) post-translational modifications of both Fos and Jun proteins that modulate their activities and (iv) interactions with other transcription factors.

Transcription factors such as AP-1 and NF-κB can be targeted for inhibition using RNA and DNA oligonucleotides acting as decoy ‘aptamers’, which bind and act as direct in vivo inhibitors. Various studies have demonstrated the potential of using specific decoy oligodeoxynucleotides (ODNs) as therapeutic or diagnostic reagents, and to dissect the specific roles of particlular transcription factors in regulating the expression of various genes (Bielinska et al., 1989; Cho-Chung et al., 1999; Eleouet et al., 1998; Jin & Howe, 1997; Mann, 1998; Morishita et al., 1997; Tomita et al., 1999; Osborne et al., 1997). Dithiophosphate oligonucleotides (S2-ODNs) and mono-thiophosphate oligonucleotides (S-ODNs), are taken up efficiently by cells and have enhanced binding to proteins (Marshall & Caruthers, 1993; Yang et al., 2002a; King et al., 2002). We previously developed thiophosphate-backbone modified aptamers (‘thioaptamers’) targeting NF-κB p50 and RelA p65 (Yang et al., 1999).

In this study, we identify the binding specificity of a thioaptamer oligonucleotide, XBY-S2, that recognizes AP-1 and can be used to modulate the basal levels of macrophage cytokine expression as well as the expression of cytokines in response to stimulation with LPS. This thioaptamer alters cytokine gene expression in macrophage cell lines as well as primary macrophages and we show that it protects animals from a potentially lethal PICV infection.

**METHODS**

**Preparation and purification of oligonucleotides.** Single-stranded oligonucleotides were synthesized to order by Bio-Synthesis Inc. Duplex oligonucleotides were annealed at 1.75 mM in 10 mM Tris/HCl (pH 7.6), 2 mM MgCl2, 50 mM NaCl, 1 mM EDTA by heating briefly at 95 °C and allowing them to cool slowly. Oligonucleotides were radiolabelled with T4 polynucleotide kinase (Promega) and [γ-32P]ATP (DuPont NEN) under standard reaction conditions. Oligonucleotides containing phosphorothioates (XBY-S2, XBY-S1 and XBY-6) were synthesized using phosphorothioamidite chemistry as previously described (Yang et al., 1999). The thiosphite triesters were oxidized with the sulfization reagent 3-ethoxy-1,2,4-dithiazoline-5-one (Xu et al., 1996). After removal of the dimethoxytrityl group, the crude oligonucleotides were purified on a Mono Q HR 10/10 FPLC column (Pharmacia) (Yang et al., 2002b). The purified oligonucleotides were desalted and concentrated by ultrafiltration using Centricon-3 (Amicon) centrifugal concentrators. Equal molar quantities of complementary strands were mixed, annealed at 90 °C and slowly cooled to form the duplexes.

**Antibodies and recombinant proteins.** Antibodies specific for ATF-2 (#222), phosphorylated ATF-2 (#9221), c-Jun (c-JunB; #9162), phosphorylated c-Jun (#9261S), CREB (#192) and phospho-thiorelated CREB (#191) were purchased from Cell Signaling Technology. Antibodies specific for c-Fos (210-128), FosB (210-114), JunD (210-111) and JunB (210-112) were purchased from Alexis Biochemicals. Antibodies directed against Fos-1 (SC183X), Fos-2A (SC171X), Fos-B (SC13017X), cAMP-responsive element modulator (CREM), JunB (SC73X), JunD (SC74X) and FosB (SC703X) were purchased from Santa Cruz Biotechnology. Antibody against c-Fos (c-FosA) was purchased from BD Biosciences and antibody to c-Jun (c-JunA) was purchased from Santa Cruz Biotechnology. Human recombinant c-Jun was purchased from Promega.

**Cell lines and culture conditions.** 702/J3 (murine pre-B lymphocyte) cells were maintained in RPMI medium supplemented with 5% fetal bovine serum (FBS), 1% β-mercaptoethanol and 2 mM glutamine. They were treated with 10 μg Salmonella enterica serovar Typhosa LPS (W0901; Difco) ml−1 for 6 h prior to extraction. P388D1 (murine monocyte-like) cells were maintained in RPMI medium supplemented with 5% FBS and 2 mM glutamine. When indicated, they were treated with 0.1 μg LPS ml−1 prior to processing for extracts. Alternatively, P388D1 cells were stimulated with poly(I:C) (25 μg ml−1; Sigma-Aldrich) in Aim V medium (Invitrogen).

**Thioaptamer treatment of P388D1 cells.** P388D1 cells were plated in T25 flasks in Aim V medium (Invitrogen). Oligonucleotide (25 μg) was mixed with 50 μl liposomes (Tfx-50, Promega) in 500 μl Aim V medium and added to the cells. Following a 2 h incubation, poly(I:C) (Sigma-Aldrich) was added to a final concentration of 25 μg ml−1 and the cells were incubated for an additional 18 h before harvesting for nuclear extract preparation. Culture supernatants were used for cytokine measurements.

**Electrophoretic mobility shift assay (EMSA).** Nuclear extracts were prepared following standard procedures as described previously (Dyer et al., 1993). For EMSA reactions, 1–5 μg nuclear extract was incubated with 0.1 pmol radiolabelled oligonucleotide in a 15 μl volume under standard reaction conditions [20 mM HEPES (pH 7.5), 50 mM KCl, 2.5 mM MgCl2, 20 mM diothiothreitol (DTT), 10% glycerol, plus 50 μg poly(dI:C) and 0.1 mg BSA ml−1] (Dyer & Herzog, 1995). For competition experiments, a 35-fold excess of unlabelled oligonucleotide was also added. For supershift experiments, nuclear extracts were incubated with antibody overnight at 4 °C in a 15 μl volume under standard reaction conditions in buffer lacking poly(dI:C) prior to the addition of the radiolabelled oligonucleotide and 50 μg poly(dI:C).

**Microaffinity isolation assay.** Microaffinity purification (MAP) of proteins binding to the AP-1 and XBY-S2 oligonucleotides was
performed by a two-step biotinylated DNA–streptavidin capture assay (Casola et al., 2000). In this assay, duplex oligonucleotides were chemically synthesized containing 5′ biotin on a flexible linker (Bio-Synthesis). Dithiolated aptamers with the biotin linker were synthesized in house. One milligram of P388D1 cell nuclear extract was incubated at 4 °C for 30 min with 50 pmol biotinylated AP-1 or XBY-S2, in the absence or presence of a 10-fold molar excess of non-biotinylated wild-type or mutated AP-1 sites. The binding buffer contained 8 μg poly(dIdC) (as non-specific competitor) and 5% (v/v) glycerol, 12 mM HEPES, 80 mM NaCl, 5 mM DTT, 5 mM MgCl2 and 0.5 mM EDTA. One hundred microlitres of a 50% slurry of prewashed streptavidin–agarose beads was then added to the sample, which was incubated at 4 °C for an additional 20 min with gentle rocking. Pellets were washed twice with 500 μl binding buffer and the washed pellets were resuspended in 100 μl 1× SDS-PAGE sample buffer. After SDS-PAGE separation, proteins were transferred to Immobilon P (Millipore) membranes for immunoblot analysis.

Immunoblots. Samples (10–20 μg) were electrophoresed by standard SDS-PAGE (8% gels) and transferred to Immobilon-P. Filters were blocked in 5% non-fat dry milk–Tris-buffered saline (TBS) overnight and then incubated with a primary antibody diluted in milk–TBS plus 100 mg BSA ml⁻¹ for 1 h. Following a series of TBS–TWEEN (0.5%) washes, the filters were incubated with horseradish peroxidase-conjugated secondary antibody in milk–TBS–BSA for 1 h. Following another series of washes, the filters were soaked in the Pierce SuperSignal chemiluminescent substrate (Pierce Biotechnology) and exposed to film.

Cytokine assays. IL-12p40, IL-12p40/p70, IL-6 and TNF-α from mouse cells were measured using ELISA kits (Biosource International) following the instructions of the manufacturer. Guinea pig TNF-α was measured by bioassay (Flick & Gifford, 1984) and guinea pig IL-8 was measured using an ELISA kit for human IL-8 (R&D Systems).

Stably transfected reporter P388D1 assays of thioaptamer action. A reporter vector containing the firefly luciferase gene with AP-1 cis-enhancer elements (pHTS-AP1) was obtained from Biomyx Technology. Control vectors had a multiple cloning site in lieu of enhancer sequences upstream of the reporter gene (designated pHTS-MCS). P388D1 cells were maintained prior to transfection in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. Cells were transfected using the calcium phosphate precipitation method and were subsequently placed in medium containing 300 μg hygromycin ml⁻¹. After 3–4 weeks, colonies were screened for the presence of the reporter gene by stimulating monolayers with 20 ng recombinant human TNF-α ml⁻¹ (R&D Systems), and assaying for luciferase activity 6–24 h later using the luciferase assay system (Promega). The P388D1 cells stably transfected with the pHTS-AP1 luciferase reporter plasmid were maintained with high-glucose DMEM containing 300 μg hygromycin ml⁻¹ and 10% FBS. For the testing of thioaptamer influence on the expression of the AP-1 driven luciferase reporter gene, 5×10⁵ P388AP1 cells were seeded into the wells of a six-well plate and grown at 37 °C overnight. XBY-S2 (10 μg per well) was added to the appropriate wells and the cultures were grown for an additional 18 h. LPS (100 ng ml⁻¹) was then added to the appropriate wells. Each control and treatment group consisted of three wells. After 6 h, the cells were rinsed twice with PBS and then lysed by the addition of 400 μl 1× lysis buffer per well (40 mM Tricine pH 7.8, 50 mM NaCl, 2 mM EDTA, 1 mM MgSO₄, 5 mM DTT and 1% Triton X-100) and incubated for 15 min at room temperature with gentle rocking. Samples were either used immediately to assay the luciferase activity or stored at −80 °C.

Virus and aptamer/liposome preparation. Pooled guinea pig spleen stock of PICV P18 virus derived from Pichinde Munchique strain (CoAn 4763) was diluted to 1000 p.f.u. ml⁻¹ in endotoxin-free PBS containing Ca²⁺ and Mg²⁺. For XBY-S2 and XBY-S1 treatment, Tfx-50 liposomes (Promega) were used as a delivery vehicle, with a constant lipid:DNA charge ratio of 1.3:1 (Ono et al., 1998) and prepared according to the manufacturer’s instructions. Aptamer/liposome was then diluted in endotoxin-free PBS containing Ca²⁺ and Mg²⁺ to a final concentration of 50 μg aptamer ml⁻¹.

Animal XBY-S2 treatment/virus inoculation. Male Hartley outbred guinea pigs, approximately 6 weeks old, were obtained from Charles River Laboratories (colony K81). Guinea pigs were treated 2 h prior to virus infection with a 1 ml intraperitoneal (ip) injection of XBY-S2 in liposomes (50 μg thioaptamer ml⁻¹). A control group received an ip injection of endotoxin-free PBS only. Two hours after the first XBY-S2/liposome injection, 1000 p.f.u. PICV P18 was inoculated ip into guinea pigs. Two days post-virus infection, guinea pigs received a second injection of thioaptamer/liposomes or PBS at the same dose as day 0. Compiled data from two separate experiments are shown, representing a total of 18 guinea pigs per group. For XBY-S1 treatment, the injections were given 1 day before infection and on days 1 and 3 post-infection, with six animals per group.

Thioaptamer treatment of primary guinea pig macrophages. Peritoneal macrophages from three male outbred Charles River guinea pigs (350 g each) were harvested by aseptic peritoneal lavage with 100 ml sterile PBS (Ca- and Mg-free). The cells were collected by centrifugation, resuspended in 2.5 ml red cell lysis buffer (0.15 M NH₄Cl, 0.1 mM Na₂–EDTA, 1.0 mM KHCO₃, pH 7.3) and placed in a 37 °C incubator for 5 min with occasional mixing. We then added 2.5 ml 1× RPMI 1640 medium supplemented with 10% (v/v) FBS, 100 U penicillin ml⁻¹, 100 μg streptomycin ml⁻¹ and 2 mM L-glutamine and plated cells into 96-well plates at a concentration of 1×10⁵ cells per well. Cells were then treated with XBY-S2 for 24 h. The supernatants were collected and analysed for TNF-α by bioassay (Aronson et al., 1995; Flick & Gifford, 1984) and IL-8 using a human IL-8 ELISA (R&D Systems) previously shown to be applicable for measuring guinea pig IL-8 (Kuo et al., 1997).

Statistics. Where indicated, statistical significance of differences between groups was determined using Student’s t-test. LogRank analysis was performed to compare survival curves for treated and untreated animals (SigmaStat 3.0; Systat Software).

RESULTS

XBY-S2 influences the outcome of lethal PICV infection

We have previously shown that increases in TNF-α have been associated with fatal PICV infection of guinea pigs (Aronson et al., 1995). Therefore, we hypothesized that inhibition of transcriptional factors would modulate the host immune response and reduce PICV-induced mortality in our guinea pig model. We have previously described utilization of systematic evolution of ligands by exponential enrichment (SELEX) technology to develop thioaptamers against several target proteins, including transcription factors (Yang et al., 2002a). Briefly, several double-stranded oligomers targeting transcription factors were tested for their ability to modulate an in vivo PICV infection. In this study we synthesized XBY-S2, a 14 bp DNA duplex that contains six dithioate residues (Table 1) (three on each
Table 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>ODN name</th>
<th>ODN sequence</th>
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<tbody>
<tr>
<td>XBY-S0</td>
<td>CCAGGTCAAGATCTG</td>
</tr>
<tr>
<td></td>
<td>GGTCCAGTCCTAGTG</td>
</tr>
<tr>
<td>XBY-S1</td>
<td>TsTsGCGGCG-A-AC-ATsG</td>
</tr>
<tr>
<td></td>
<td>A-A-CGCCGGsTsGTA-T</td>
</tr>
<tr>
<td>XBY-S2</td>
<td>CCA-Tg-ACTTCA-AGTsG</td>
</tr>
<tr>
<td></td>
<td>GGA-TCA-CGTA-GTsGTA-T</td>
</tr>
<tr>
<td>XBY-6</td>
<td>CC-AGG-AG-ATsTCC-AC</td>
</tr>
<tr>
<td></td>
<td>GGA-TCCsTcsTA-A-GGsTG</td>
</tr>
<tr>
<td>AP-1</td>
<td>CGTCTGATGCTCAAGCGAA</td>
</tr>
<tr>
<td></td>
<td>GCGAACTACTGAGTTGCTTT</td>
</tr>
<tr>
<td>CREB</td>
<td>AGAGATTGCCGAGCTCAAGAGAGTAG</td>
</tr>
<tr>
<td></td>
<td>TCTCTAAGGAGTCAGCTAGTCTCGA</td>
</tr>
<tr>
<td>NF-kB</td>
<td>AGTTGAGGGGACCTTTCCAGGC</td>
</tr>
<tr>
<td></td>
<td>TCAACTCCCCCTGAAAGGTCC</td>
</tr>
<tr>
<td>ISRE</td>
<td>GATGGGAAAACCGAAAAGCTAGG</td>
</tr>
<tr>
<td></td>
<td>CTA GCCCTTGCGCTTGGACTTGG</td>
</tr>
</tbody>
</table>

‘s’ denotes a dithioated linkage. Binding sites are in bold.

Fig. 1. XBY-S2 prolongs and increases survival of PICV-infected guinea pigs. (a) Guinea pigs were treated with XBY-S2 (solid line) at 2 h prior to infection with a lethal dose of PICV variant P18. A control group received an injection of endotoxin-free PBS only (dashed line). Mortality was monitored for 40 days post-infection. (b) XBY-S1 (solid line) decreases survival; treatment was at -1, 1 and 3 days post-infection.

EMSA analysis of P388D1 and 70Z/3 cells

Since XBY-S2 proved to be effective against PICV infection in guinea pigs, we sought to investigate the mechanism of action and binding specificity of XBY-S2. An examination of the XBY-S2 sequence revealed an AP-1 consensus binding site TGA(C/G)TCA that resembled the related CRE-binding site, TGACGTCAG. The binding specificity of the XBY-S2 oligonucleotide was determined using lysates from mouse P388D1 macrophages and 70Z/3 mouse pre-B cells. 32P-labelled XBY-S2 was found to bind specifically with proteins in the extracts, forming one discrete band (Fig. 2a). Fully phosphorothioate-substituted oligonucleotides are unable to show binding specificity in this assay due to very high levels of non-specific binding. Only a subset of the partially dithioate-substituted oligonucleotides show binding, depending on the sequence specificity of their binding sites. The XBY-S2 binding was successfully competed by unlabelled XBY-S2, indicating specific binding, and by AP-1 and CREB oligonucleotides, indicating that the binding was to proteins in the AP-1 family. Neither an oligonucleotide with an NF-kB nor an interferon-stimulated response element (ISRE)-binding site was capable of competing for binding, indicating that these immune-associated transcription factors are not bound by XBY-S2 (Fig. 2a). In the reciprocal binding assays, binding of AP-1 proteins by their consensus oligonucleotide was successfully competed by XBY-S2, confirming that XBY-S2 was binding to the same proteins as the AP-1 consensus oligonucleotide (Fig. 2b). XBY-S2 was also specifically bound by human recombinant AP-1 and c-Jun protein dimers (data not shown).
Identification of the AP-1 complexes binding to the AP-1 and XBY-S2 oligonucleotides

There are more than 10 different members of the AP-1 family of proteins, which dimerize in various combinations. To determine which of the AP-1 proteins were binding to the XBY-S2 oligonucleotide, we used a combination of immunoblots, supershift EMSAs and MAP. Immunoblots revealed that P388D1 cells contain JunB, c-Jun, Fra-2, CREB, CREM and possibly JunD proteins (Fig. 3a). High background in immunoblots using anti-Fra-2(A) and anti-c-Fos(A) precluded their use and inclusion in the selection shown in Fig. 3(a). Subsequently, supershift EMSA reactions were used to determine which of the AP-1 proteins were binding to XBY-S2 on the EMSA gels (Fig. 3b). Where possible, we used more than one antibody from different sources, or specific to different portions of an AP-1 protein, to confirm these results. Supershift analysis revealed that AP-1 complexes in stimulated P388D1 cells contained detectable levels of Fra-2, c-Fos, JunB and JunD, as well as c-Jun and phosphorylated c-Jun (Fig. 3b). We were unable to detect the presence of active Fra-1, FosB, ATF2, CREM and CREB using supershift assays. Most of the AP-1 complexes contain Fra-2 and/or JunB since the combination of antibodies can supershift the majority of the complexes (data not shown). The same experiment was performed to identify the AP-1 proteins that bind to XBY-S2 (Fig. 3b). Fra-2, c-Fos, JunB and possibly c-Jun were found in complexes that bound XBY-S2.

Fig. 2. XBY-S2 binds to AP-1 proteins in both macrophage and B cell lines. EMSA analysis to determine the complexes present in mouse 70Z/3 pre-B cells (activated with LPS) and P388D1 mouse macrophage-like cells [activated with poly(I:C)] using either a consensus AP-1 binding site (a) or XBY-S2 thioaptamer (b). Competition with 50-fold molar excess unlabelled transcription factor-binding site oligonucleotides (AP-1, CREB, XBY-S2, NF-kB and ISRE) in the EMSA reaction was performed as indicated.

Fig. 3. Determination of AP-1 proteins capable of binding to the XBY-S2 thioaptamer decoy. Abbreviations: pc-Jun, phosphorylated c-Jun; pATF-2, phosphorylated ATF-2; pCREB, phosphorylated CREB. (a) Immunoblot analysis of P388D1 nuclear extracts with the indicated antibodies for AP-1/CREB family members. Two separate antibodies for Fra-2, c-Jun and c-Fos were used and are distinguished by the A and B designation. (b) Supershift analysis of P388D1 nuclear protein complexes binding to either the AP-1 consensus oligonucleotide or the XBY-S2 thioaptamer. Antibodies are the same as those used for immunoblots and were added to each of the EMSA reactions to achieve a supershift as indicated. Supershifted bands are indicated by a dot. (c) MAP of proteins in P388D1 nuclei which bind to the XBY-S2 aptamer was followed by immunoblot analysis of the proteins that were bound.
Due to the limited availability of antibodies that are effective in supershift analysis, we also used MAP in conjunction with immunoblotting to confirm the results of negative supershift data. MAP is a more sensitive assay than either immunoblotting or EMSA. XBY-S2 was end-labelled with biotin and streptavidin beads were used to purify any proteins that were bound to it. MAP results established that XBY-S2 was recognized by AP-1 dimers that also included CREM, but there was still no detectable ATF-2 or FosB (Fig. 3c). Either the complexes containing CREM are not detectable by EMSA, or the CREM antibody is not effective when used in the EMSA supershift protocol, but is effective in immunoblot analysis.

Treatment of P388D1 cells with XBY-S2 eliminates AP-1 DNA-binding activity

XBY-S2 includes a consensus AP-1-binding site [5'-TGA(G/C)TCA-3'] and, when used as a decoy aptamer, it would be expected to influence the expression of cytokines from macrophages by acting to sequester the AP-1 proteins in cells. In order to demonstrate that XBY-S2 functions as a decoy, P388D1 macrophage cultures were treated in triplicate with liposomes, either with or without XBY-S2, for 2 h prior to stimulation with LPS; liposomes were used to deliver the thioaptamer into cells more efficiently. XBY-S0 was used as a control oligonucleotide since it contains the same base composition as the XBY-S2 oligonucleotide, though it does not contain any dithioate modifications. Nuclear extracts were harvested at 16 h post-stimulation with LPS and analysed by EMSA. The EMSA gels were quantified, to allow statistical analysis, and the results are depicted graphically in Fig. 4(a). Treatment of cells with XBY-S2 significantly reduced the AP-1-binding activity. Treatment with the control oligonucleotide XBY-S0 led to a non-significant reduction of AP-1-binding activity and was significantly higher than the levels of AP-1 in the XBY-S2-treated cells. Therefore, the XBY-S2 thioaptamer appears to be an efficient and specific inhibitor of AP-1 transcription factor DNA-binding activity.

Reporter assays confirm that XBY-S2 treatment of P388 cells inhibits AP-1 transcription factors activity

In order to confirm that the XBY-S2 thioaptamer altered AP-1 regulated transcription, we established a P388D1-derived cell line (P388AP1) stably transformed with an AP-1 driven luciferase reporter plasmid, pHTS-AP1 (BioMyx Technology). This plasmid contains a tandem repeat of six copies of an AP-1 site (TGACTAA) linked to the luciferase gene. Fig. 4(b) illustrates that the P388AP1 reporter cell line responds to LPS stimulation with a twofold increase in the expression of luciferase. Treatment with XBY-S2 alone stimulated nearly the same increase in luciferase expression as LPS stimulation. LPS in addition to XBY-S2 resulted in an additive increase of luciferase expression. Treatment with a thioaptamer without an AP-1 site had no effect on AP-1-driven expression of luciferase (data not shown). Thus, XBY-S2 appears to function by increasing AP-1-driven transcription, possibly by blocking Fra-2, the repressive AP-1 subunit that is present in large amounts in these cells.

XBY-S2 perturbation of cytokine expression in P388D1 cells in response to poly(I:C)

Poly(I:C) is a potent inducer of cytokine expression through Toll-like receptors (Alexopoulou et al., 2001). To determine whether XBY-S2 can influence cytokine gene expression, cytokines elaborated from macrophages following poly(I:C) stimulation were measured in combination with prior treatment with the XBY-S2 thioaptamer. As above, liposomes were used to deliver the aptamer more efficiently. A 2 h pre-treatment with XBY-S2 increased the expression of IL-12p40 + p70 (sixfold), IL-6 (3.5-fold), and TNF-α (1.7-fold) over the levels expressed in cells stimulated with poly(I:C) alone (Fig. 5). IL-12p70 was only detectable in those cells treated with XBY-S2 and poly(I:C). Interestingly, liposome treatment without thioaptamer appeared to
suppress cytokine gene expression under these conditions. These data suggest that either binding of AP-1 proteins to the XBY-S2 decoy aptamer eliminates transcriptional repressors of these cytokines, or a specific set of AP-1 proteins regulates the expression of another protein that serves as a repressor. These data also suggest that XBY-S2 modulation of AP-1 proteins alters the expression of pro-inflammatory cytokines that could influence innate immunity induced in response to viral challenge.

**XBY-S2 perturbation of cytokine expression in primary guinea pig macrophages**

To determine whether XBY-S2 can also influence cytokine gene expression in primary cells from guinea pigs, the cytokine production from primary peritoneal macrophages was examined following treatment with the XBY-S2 thioaptamer. Due to the limited availability of anti-guinea pig-based reagents, we are limited as to the cytokines that can be measured in the guinea pig. The supernatants of cultured primary guinea pig peritoneal macrophages were assayed for TNF-α and IL-8 was measured by ELISA based on human IL-8. These two cytokines were chosen because they are among the few guinea pig macrophage-derived cytokines for which available standardized assays exist, and because AP-1 is known to be important in regulation of their transcription (Hoffmann et al., 2002). XBY-S2 increased the expression of IL-8 in a dose-dependent fashion over the levels expressed in untreated macrophages (Fig. 6). At doses below 20 μg per 10^7 cells, XBY-S2 appeared to increase basal level of TNF-α expression, but there was no clear dose dependence at higher doses. Therefore, these data, in part, confirm the results seen in the P388D1 cell line, which indicate that XBY-S2 either directly targets AP-1 proteins that repress the transcription of these cytokines, or AP-1 regulates the expression of another protein that serves as a repressor. XBY-S2 does not block the activities of transcription factors that activate the expression of these cytokines. However, XBY-S2 increases the basal level of expression of pro-inflammatory cytokines that could influence innate immunity induced in response to viral challenge.

**DISCUSSION**

PICV infection of guinea pigs causes a syndrome that is indicative of immune system malfunction (Aronson et al., 1995; Peters et al., 1989). Infection of guinea pigs with the virulent P18 variant is characterized by dysregulated...
pro-inflammatory cytokine production (Aronson et al., 1995) with profound terminal shock. The macrophage tropism of PICV is a characteristic of many haemorrhagic fever viruses (reviewed by Peters et al., 1989) and may be an important contributor to either the ability of the virus to evade the immune system and/or the eventual fatal shock syndrome. Virus infection of macrophages presents an opportunity for a virus to evade the immune system by modulating the immune response of the infected cell.

Inhibition of macrophage activation would favour viral spread, as activation of macrophages is known to increase the microbicidal action of these phagocytes (Oswald et al., 1992). Therefore, treatment with an immunomodulating agent such as the thioaptamer XBY-S2 may alter the expression of a number of cytokines that are important in generating an effective early innate response, in the development of the protective Th-1 type antiviral response (Fennewald et al., 2002). We previously described that the attenuated PICV P2 variant is associated with the appearance of activated forms of transcription factors NF-κB in macrophages, while the lethal PICV P18 variant is associated with the ‘non-activated’ state of these transcription factors that appears to correlate with a failure to mount an effective immune response (Fennewald et al., 2002). Here we demonstrate that modulation of the DNA-binding activity of another transcription factor, AP-1, appears to eliminate the repression of cytokine gene expression, elevating the basal and, in some circumstances, the induced cytokines. Modulation of AP-1 is capable of protecting guinea pigs from lethal PICV infection and we hypothesize that this is the result of changes in host cell gene expression that counteract inhibitions by pathogenic PICV.

Though we were somewhat surprised to find that our most active aptamer was binding to AP-1, it is known that AP-1 is important in the immune response (Adcock, 1997). Although there are over 50 members of the AP-1 family of proteins and potentially over 1000 different homo- and heterodimeric forms (Newman & Keating, 2003), the majority of the detectable AP-1 DNA-binding activity in mouse macrophages consists of dimers that include Fra-2 and JunB. As Fra-2 can also negatively regulate c-Jun activity (Suzuki et al., 1991; Sonobe et al., 1995) and can form heterodimers with JunB that act as transcriptional repressors in keratinocytes (Rutberg et al., 1997), it is conceivable that the negative influence of c-Jun and JunB on basal cytokine expression in macrophages may arise from formation of repressive Jun/Fra-2 heterodimers. The treatment of macrophages with the thioaptamer XBY-S2 can completely abrogate AP-1 DNA binding activity and hence could be acting as a decoy to effectively outcompete the repressive Jun/Fra-2 dimers. By modulating the activity of AP-1 we have shown that we can increase the expression of several key cytokines including TNF-α, IL-8, IL-6 and IL-12 and increase the survival of guinea pigs to lethal PICV infection.

The increased expression of IL-12 may be particularly important. It is consistent with the inhibitory action of some AP-1 dimers and the work of Roy et al. (1999), who demonstrated that a deficiency in c-Fos increased macrophage IL-12 production. Previous research has demonstrated that IL-12 administration at low doses can be efficacious in mice infected with LCMV, resulting in inhibition of virus replication and enhanced CD-8 responses (Orange et al., 1995). Similarly, low doses of IL-12 are also effective in protecting mice against Encephalomyocarditis virus, Murine hepatitis virus and herpes simplex virus, and promotes clearance of vesicular stomatitis virus while having other positive effects on the outcome of a number of other virus infections (Komastu et al., 1998). Though we are unable to confirm the observations in vivo in PICV-infected guinea pigs due to the lack of antibody reagents or a bioassay, the increase in IL-12 production following XBY-S2 treatment could contribute to the efficacy in the animal.

As with all treatments, it is not entirely proven that the in vivo efficacy is due to the measured in vitro activity – that the ability of XBY-S2 to prolong survival is due to its in vitro ability to inhibit AP-1 proteins. While phosphorothioate oligonucleotides are known to give strong non-specific effects, our modestly modified aptamers continue to show specific binding in assays and only the XBY-S2 aptamer showed significant protection in the animal model. Still, we cannot rule out the possibility that additional activities, including Toll-like receptor responses, are important in the in vivo efficacy. The XBY-S2 activity increases the interest in the AP-1 proteins and their significance in both positive and negative regulation of the immune response.

Inhibition of AP-1 with XBY-S2 treatment increases cytokine gene expression and protects against virus infection. It is of interest to note that Lassa virus infection of macrophages leads to the release of virus particles, but not to an increase in TNF-α, IL-1β, IL-12p35 and p40, IL-10, IL-6, TGF-β, IFN-γ or CD25 synthesis (Baize et al., 2004). Interestingly, these authors also demonstrated that Lassa virus infection of dendritic cells led to the expression of elevated levels of IL-8 but no other chemokines. Therefore, our data using virulent PICV correlate with results from studies with Lassa virus that indicate that there is viral suppression of the normal response of macrophages and dendritic cells to dsRNA or viral infection.

ACKNOWLEDGEMENTS

We wish to express our deep regrets at the death of Dr Robert E. Shope on 19 January 2004 in Galveston and to acknowledge his leadership and participation in this research. Dr Shope had a world-class career of more than 40 years of identifying and isolating infectious diseases worldwide. His contributions to the scientific arena are extensive, but more importantly, he consistently exemplified and fostered an attitude.
of service and collegiality. Dr Shope was admired and beloved by students and scientific colleagues alike, and his contributions to science and society will long be remembered. We wish to thank Barry Elsom for his excellent technical assistance and Dr Debra Kallick for her helpful discussions. This work was supported by grants from DARPA (DAAD19011037), DTRA (DAAD17-01-D0001), NIH (N01-HV-28184, U01 AI054827, R01 AI27744) and by a grant from NAID to Dr Herzog through the Western Regional Center of Excellence for Biodefence and Emerging Infectious Disease Research, NIH grant number U54 AI057156.

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