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The role of leukocyte function-associated antigen-1 in animal models of inflammation

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Abstract

Both preclinical and clinical data have identified leukocyte function-associated antigen-1 (LFA-1) as an important component of inflammatory disease states. We evaluated small molecule inhibitors of this glycoprotein in several animal models in which the inflammatory process is dependent on human or non-human primate LFA-1. (*R*)-5(4-bromobenzyl)-3(3,5-dichlorophenyl)-1,5-dimethylimidazolidine-2,4-dione, BIRT 377, effectively suppressed the production of human immunoglobulin (IgG) following reconstitution of severe combined immunodeficient (SCID) mice with human peripheral blood mononuclear cells. The BIRT 377 analog, BIX 642, inhibited the cellular infiltrate and increase in skin thickness associated with the delayed-type hypersensitivity reaction in previously immunized squirrel monkeys challenged with antigen. BIX 642 also inhibited the trans-vivo delayed-type hypersensitivity response in the footpads of SCID mice injected with human peripheral blood mononuclear cells and donor-sensitive antigen. These results demonstrate the efficacy of small molecule inhibitors of LFA-1 in preclinical models of inflammation dependent on human or non-human primate LFA-1.

Keywords: Hypersensitivity, delayed-type; LFA-1 (leukocyte function-associated antigen-1); Trans-vivo; Inflammation; Staphylococcal enterotoxin B; T cell; Antigen presentation; SCID, mouse; Leukocyte; Cell adhesion

1. Introduction

Leukocyte trafficking and activation are essential for the varied host defense mechanisms targeting elimination of foreign invaders, but, as a consequence of either a misdirected or fulminating mode, are also identified as important instigators in most acute and chronic disease states. Trafficking of granulocytes with subsequent release of destructive oxygen radicals and proteases has been implicated in the pathophysiological sequelae during the reperfusion of ischemic tissue in transplantation, stroke and myocardial infarction and is believed to underlie the chronic pulmonary dysfunction in asthmatics (Kishimoto and Rothlein, 1994; Bryan et al., 2000). T lymphocytes recognizing auto or "self" antigens are believed to be important in the triggering of autoimmune syndromes which involves activation of macrophages and B lymphocytes for orchestrating the cellular and humoral arms, respectively, of the effector stage in tissue destruction (Bach, 1993). Abnormal activation of resident inflammatory cells is also implicated in the pathology of pulmonary syndromes such as severe asthma and Chronic Obstructive Pulmonary Disease (Wenzel et al., 1997; Leckie et al., 2000). Therefore, blockade of trafficking and/or activation of leukocytes has been pursued rigorously as drug discovery projects in the pharmaceutical industry.

Cellular adhesion glycoproteins have been recognized as key molecules in regulating both the trafficking and activation of leukocytes (Springer, 1994; Kishimoto and Rothlein, 1994). The leukocyte function-associated family of integrins (CD11/CD18, LFA or β2 integrin family) is widely and uniquely expressed on leukocytes and subserves important functions for the trafficking and activation of these cells. Analysis of patients presenting with recurrent bacterial infections led to the identification of CD18, the common β chain of the LFA family of integrins, as the molecular basis underlying decreased leuko-

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cyte adhesion capacity (Arnaout et al., 1982; Springer et al., 1984). Among these family members, leukocyte function-associated antigen-1 (LFA-1; CD11a; αLβ2) has emerged as a multitask surface molecule, contributing to trafficking patterns of marginating leukocytes but also effecting costimulatory signals during the activation of T lymphocytes by antigen presented in the context of major histocompatibility complex Class II by antigen presenting cells. The involvement of LFA-1 in leukocyte trafficking is reflected in the effectiveness of antibodies to this glycoprotein in limiting the neutrophil infiltration into inflamed cavities. The importance of LFA-1 in leukocyte activation is substantiated by the efficacy of these antibodies in prolonging the life of allograft transplantation in both preclinical and clinical settings (Isobe et al., 1992; Harrison and Madwed, 1999; Poston et al., 2000; Hourmant et al., 1994), in models of autoimmune syndromes such as type 1 diabetes (Hasegawa et al., 1994) and in clinical trials of the autoimmune disease psoriasis (Gottlieb et al., 2000). Targeted deletion of LFA-1 in mice results in a phenotype of decreased lymphocyte recirculation, decreased leukocyte adhesion to the ligand intercellular adhesion molecule (ICAM)-1, and decreased delayed-type hypersensitivity reactions (Schmits et al., 1996; Shier et al., 1996; Andrew et al., 1998). For comparison, the family member Mac-1 (CD11b; αMβ2) has a more limited distribution among leukocytes (Kishimoto and Rothlein, 1994) and genetic depletion primarily affects neutrophil function (Coxon et al., 1996). The principle ligands for LFA-1 are ICAM-1, ICAM-2 and ICAM-3 (Rothlein et al., 1986; Kishimoto and Rothlein, 1994).

The efficacy of antibodies to LFA-1 in animal models of inflammation, and in clinical trials, has laid a proof-of-principle foundation for this glycoprotein as a validated drug discovery target. Recently, a small molecule inhibitor of LFA-1 was developed in our research laboratories (Kelly et al., 1999). The compound, BIRT 377, potently and selectively inhibits the binding of LFA-1 to ICAM-1 without affecting the interaction of ICAM-1 with Mac-1. Specifically, binding to LFA-1, and not Mac-1, is anticipated to affect leukocyte trafficking and activation while maintaining innate inflammatory responses to bacterial infection, since Mac-1 is the primary receptor for neutrophil phagocytosis of opsonized bacteria.

During the course of evaluating BIRT 377 and its analogues, it became clear that many of these compounds exhibited a predilection for inhibiting human versus rodent LFA-1. Therefore, it became necessary to develop animal models, which would allow the evaluation of compounds on human, and possibly non-human primate LFA-1. In this pursuit, we assessed the efficacy of compounds in rodent models, which involves the incorporation of human blood leukocytes in mediating the functional response. In addition, we developed a model of delayed-type hypersensitivity in non-human primates, to assess the role of LFA-1 in this basic inflammatory response which is a component of graft rejection, tumor immunity and many autoimmune syndromes. The efficacy of these compounds in these various models was compared to that found with an antibody to LFA-1.

2. Materials and methods

All experimental protocols were reviewed and approved by the Animal Care and Use Committee at Boehringer Ingelheim Pharmaceuticals.

2.1. Human immunoglobulin G (IgG) production in severe combined immunodeficient (SCID) mice

Male NOD/LtSz SCID/SCID mice were obtained from The Jackson Laboratories and housed under 12-h light/dark cycles with free access to food and water. On the experimental day, human peripheral blood mononuclear cells were obtained from blood samples drawn from the in-house donor program. Briefly, 100 ml of heparinized blood was layered over Ficoll–Paque with the mononuclear cell interface collected, washed and resuspended in phosphate-buffered saline (PBS). Mice were injected intraperitoneally with $3–10 \times 10^7$ cells in 500 μl of buffer (day 0). Isolated peripheral blood mononuclear cells from each donor were halved for injection into a control and treated animal. Serum samples were collected weekly for evaluation of human IgG. Mice were treated with either 100 μg (i.p.) of antibody on days −1, 0 and 1, or with BIRT 377 (3 and 10 mg/kg oral with olive oil as vehicle) on days 0 through 14.

Human IgG was evaluated using an enzyme-linked immunosorbent assay. Dynatech Flex plates were coated with goat, anti-human IgG FAB2 (Jackson, 109-006-098) in phosphate-buffered saline overnight at 4 °C. Following the washing of the plates, serum samples were added (50 μl/well) for 1.5 h followed by washing and the addition of biotin-labeled goat-anti-human IgG (Jackson, 1030-08; 1/25,000 dilution in phosphate-buffered saline/tween) for 1 h at room temperature. Plates were again washed and horseradish peroxidose–strepavidin (Jackson) was added at a 1/2000 dilution for 1 h. Substrate was then added for color development.

2.2. Squirrel monkey model of delayed-type hypersensitivity

Squirrel monkeys (*Saimiri sciureus*, 0.6 to 1.3 kg) were obtained and housed in our animal facilities under 12-h light/dark cycles with free access to food and water. Animals were immunized with live Bacillus of Calmette and Guerin (BCG) (1.3×10^7 CFU s.c. and i.d.) in PBS approximately 3–6 months prior to experimentation. For conducting the delayed-type hypersensitivity reaction, animals were challenged with four intradermal injections (100

μl) of either phosphate-buffered saline or purified protein derivative of Mycobacterium bovis (PPD, 30 μg). Animals were dosed with either an anti-LFA-1 (R3.1, 3 mg/kg i.v., qd) or with an analog of BIRT 377, BIX 642 (100 mg/kg p.o., bid), beginning 48 h prior to PPD challenge. Control animals received the vehicle for either BIX 642 (30% cremophore, 2.5 ml/kg, bid) or the anti-LFA-1 (PBS, 2.5 ml/kg). Skin thickness was measured with a digital caliper. Skin biopsies were occasionally isolated for histological analysis.

Blood samples from each monkey were evaluated in a whole blood interleukin-2 assay prior to the animal being enrolled in a delayed-type hypersensitivity protocol. Briefly, heparinized blood was aliquoted into 96-well plates and incubated overnight at 37 °C in the presence of staphylococcal enterotoxin B (200 ng/ml). Plates were spun ($700 \times g$ for 10 min) and 50 μl of serum collected for determining levels of interleukin-2 (Biosource Rhesus Monkey IL-2 Cytoscreen enzyme-linked immunosorbent assay kit). Developed samples were read for optical density (450 nm) on a microplate reader (Molecular Devices).

2.3. Trans-vivo model of delayed-type hypersensitivity

Trans-vivo delayed-type hypersensitivity was performed based on the model by Carrodeguas et al. (1999). SCID mice (C57Bl/6 background) were obtained from The Jackson Laboratories and housed under 12-h light/dark cycles with free access to food and water. On the experimental day, human peripheral blood mononuclear cells were isolated from blood samples drawn from donors previously screened for positive in vitro proliferation reactions to tetanus antigen. One hundred microliters of whole blood was collected into Cell Preparation Tube Vacutainer tubes (Becton Dickinson, NJ), spun at 1800 RCF for 30 min, and the buffy layer was separated, washed and counted. $7–10 \times 10^6$ peripheral blood mononuclear cells mixed with 25 lf units of tetanus toxoid (TT; Tetguard, BI-Vetmedica) in a volume of 25–40 μl were injected into the hindlimb footpads of mice. Foot pad thickness was measured both pre-injection and 24-h post-injection with a dial thickness gauge (Mitutoyo). Pre-injection thickness values were subtracted from the readings post-injection to obtain the change in paw thickness.

Mice were dosed orally with the BIRT 377 analog, BIX 642 (30 mg/kg, p.o.) at $t = -1$, 4 and 19 h relative to the time of paw injection. Anti-human (R7.1) or anti-mouse (M17/4) LFA-1 was administered, at 5 mg/kg i.p., 1 h prior to paw injection. Each treatment group was comprised of four donors with the peripheral blood mononuclear cells from each donor being split and evaluated in two mice.

2.4. Reagents

The small molecule antagonists and anti-human antibodies to LFA-1 (R7.1 and R3.1) were generated internally as previously reported (Kelly et al., 1999). 1B7.11 (control IgG) and M17/4 (anti-mouse LFA-1 monoclonal antibody) were generated from cell lines obtained from American Type Culture Collection (Rockville, MD).

2.5. Data analysis

Comparisons for differences were done with Analysis of Variance (ANOVA) followed by the least squares means test or Kruskal–Wallis ANOVA on ranks followed by Dunn's test for differences between individual means ($P < 0.05$).

3. Results

3.1. Human IgG production in SCID mice

Reconstitution of SCID mice with human peripheral blood mononuclear cells leads to a xenoreactive production of human IgG against mouse erythrocytes (Abedi et al., 1992). Production of human IgG was observed beginning 1–2-weeks post-reconstitution and peaked between weeks 5 and 6 (Fig. 1). Administration of an anti-LFA-1 (R3.1; 100 μg i.p. for 3 days) caused a near-complete inhibition of the human antibody response (Fig. 1A). Dos-

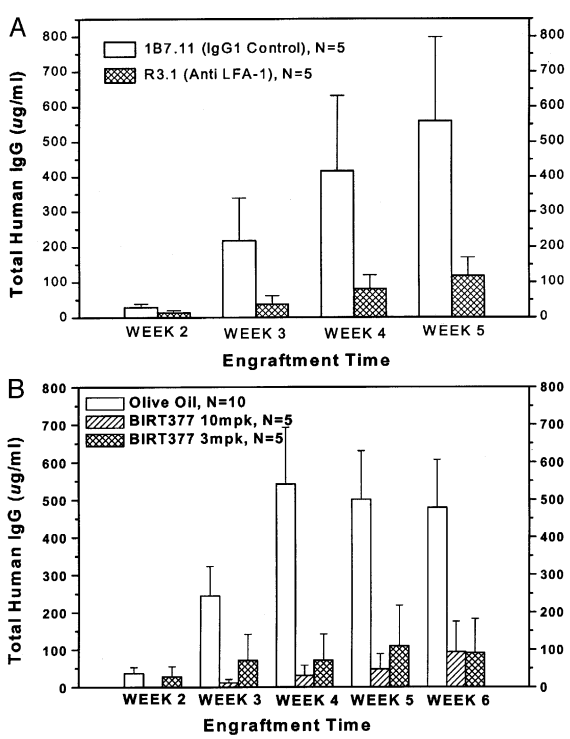

Fig. 1. Production of human IgG in NOD/LtSz SCID mice reconstituted with human peripheral blood mononuclear cells. (A) Production of human IgG in animals dosed with anti-LFA-1 (R3.1, 100 μg at days −1, 0 and 1 relative to reconstitution) or with an isotype control antibody (1B7.11, same dosing regimen). (B) Production of human IgG in the presence of BIRT 377 (3 or 10 mg/kg dosed daily for 2 weeks) or vehicle (olive oil).

ing animals with the small molecule antagonist of LFA-1, BIRT 377 also prevented the human antibody response (Fig. 1B). This effect occurred with both the 3- and 10-mg/kg dosing regimens.

3.2. Squirrel monkey delayed-type hypersensitivity

Squirrel monkeys challenged with PPD, following immunization with BCG, exhibited a delayed-type hypersensitivity reaction as evidenced by an increased skin thickness at sites of the PPD challenge (Fig. 2) and by a robust inflammatory cell infiltrate (data not shown). The delayed-type hypersensitivity response was significantly inhibited in squirrel monkeys receiving anti-LFA-1 (R3.1, 3 mg/kg i.v., qd). Dosing animals with the BIRT 377 analog, BIX 642 (100 mg/kg, bid) also led to a significant inhibition of the delayed-type hypersensitivity response. Tissue sections from the animals treated with either the anti-LFA-1 or BIX 642 showed decreased inflammatory cellular infiltration (data not shown).

Both an anti-LFA-1 (R7.1, $92 \pm 0.2\%$ at 10 μg/ml) and BIX 642 ($IC_{50} = 18 \pm 14$ nM, $n = 4$) effectively inhibited the production of interleukin-2 in response to staphylococcal enterotoxin B in squirrel monkey whole blood.

3.3. Trans-vivo delayed-type hypersensitivity

Injection of human peripheral blood mononuclear cells, with donor-sensitive antigen, into either SCID or normal mouse strains leads to a delayed-type hypersensitivity-like swelling response termed trans-vivo delayed-type hypersensitivity (Carrodeguas et al., 1999). Fig. 3 shows this trans-vivo delayed-type hypersensitivity response in C57Bl/6 SCID mice injected with human peripheral blood mononuclear cells. Cells alone showed no apparent reaction as evaluated by paw swelling or in tissue histology (data not shown). Injection of the human peripheral blood mononuclear cells mixed with donor-sensitive antigen as determined by prior cell-based proliferation assays caused a robust reaction as measured by an increase in paw swelling at 24 h. Dosing animals with either anti-human (R7.1, 5 mg/kg i.p.) or anti-mouse (M17/4, same dosing regimen) LFA-1 caused significant decreases in the trans-vivo delayed-type hypersensitivity reaction. The trans-vivo delayed-type hypersensitivity reaction was also inhibited if animals were dosed with the BIRT 377 analog, BIX 642 (30 mg/kg, dosed at 1-h pre-, 4- and 19-h post-administration of human peripheral blood mononuclear cells).

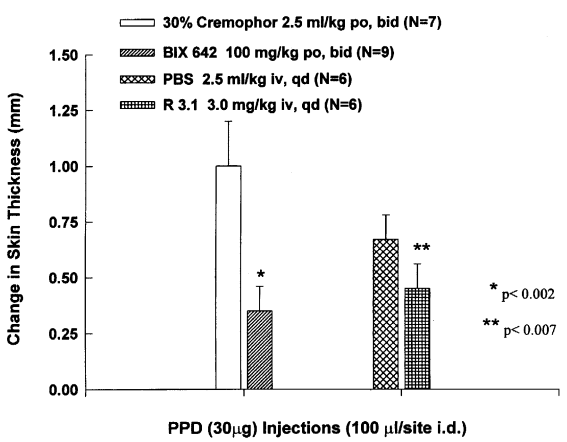

Fig. 2. Delayed-type hypersensitivity response in squirrel monkeys (*S. sciureus*). Monkeys were immunized with BCG and challenged, intradermally, with either phosphate-buffered saline or PPD (30 μg). Animals dosed with the vehicle for either BIX 642 (30% cremophore) or for R3.1 (PBS) exhibited a delayed-type hypersensitivity reaction manifest by an increased skin thickness at sites of PPD challenge. Dosing animals with either anti-human (R3.1, 3 mg/kg i.v. daily) or BIX 642 (100 mg/kg p.o., bid) caused a significant inhibition of the delayed-type hypersensitivity response.

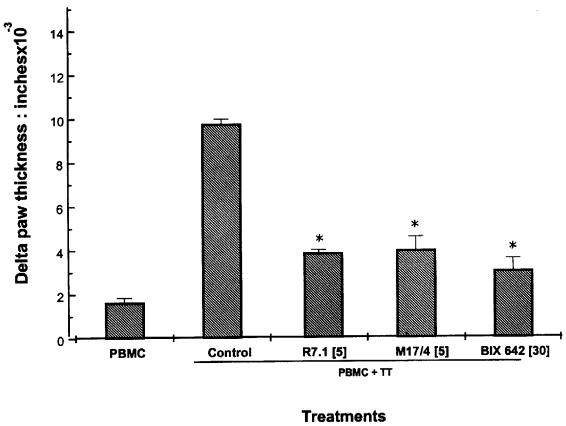

Fig. 3. Trans-vivo delayed-type hypersensitivity response in C57Bl/6 SCID mice. Human peripheral blood mononuclear cells were injected into footpads with swelling monitored at 24-h post-injection. Each bar represents the mean (±S.E.M.) of responses from four donors, with each donor value representing the average from two mice (i.e., two mice were injected per donor). Peripheral mononuclear cells with antigen (25 lf units of tetanus toxoid) caused a robust reaction compared to peripheral mononuclear cells alone. Dosing animals with either human (R7.1, 5 mg/kg i.p. at 1-h pre-administration of peripheral mononuclear cells) or mouse (M17/4, same dosing regimen) anti-LFA-1 caused a statistically significant decrease in the delayed-type hypersensitivity reaction. Dosing animals with BIX 642 (30 mg/kg p.o., 1-h pre, 4- and 19-h post) caused a comparable, significant reduction in the trans-vivo delayed-type hypersensitivity response. $^{*}P < 0.05$ compared to vehicle analysis of variance.

4. Discussion

Studies utilizing antibodies to LFA-1 have demonstrated, both preclinically and clinically, that this glycoprotein is an attractive target for drug discovery. Recently, a small molecule antagonist of LFA-1 has been discovered which should lead to effective, orally active compounds for clinical syndromes, which harbor both acute and chronic inflammatory events. This pursuit has benefited from the evaluation of such compounds in preclinical models in

which the inflammatory event is dependent on human, or non-human primate LFA-1.

We have found that the recently discovered LFA-1 antagonists, BIRT 377 and BIX 642, exhibit impressive efficacy in models based on primate LFA-1. BIRT 377 potently inhibited the production of human IgG in SCID mice engrafted with human peripheral blood mononuclear cells (Fig. 1). The efficacy of BIRT 377 was comparable to that achieved with an antibody to LFA-1, and was apparent up to 6-weeks post-injection of the human peripheral blood mononuclear cells. The dosing regimen employed (once daily for 2 weeks) may have demonstrated efficacy by preventing successful engraftment of the human cells, possibly by inhibiting cognate interactions of the human lymphocytes (Janeway and Travers, 1997). The inhibitory effect of BIRT 377, or the anti-LFA-1 antibody, was unlikely due to a direct toxic effect since no sign of cellular toxicity has been observed upon incubation of human cells with these reagents in vitro (P. Reilly, unpublished observations; Kelly et al., 1999). It is of interest to note that decreased immunoglobulin production was a phenomenon observed in the leukocyte adhesion deficient patients (Anderson et al., 1985).

Evaluation of the small molecule compounds in T-cell-dependent inflammatory responses was conducted in models of delayed-type hypersensitivity. In squirrel monkeys, the BIRT analog BIX 642 decreased both the tissue-swelling response to injected antigen (Fig. 2) as well as the histological score of mononuclear infiltrate in skin biopsies. Delayed-type hypersensitivity reactions involve many of the cellular interactions and cytokine components believed to be instrumental for the tissue destruction in autoimmune diseases (Janeway and Travers, 1997). Efficacy in the squirrel monkey model may be a consequence of either inhibiting T-cell activation (i.e., inhibition of LFA-1/ICAM-1 costimulatory signaling) or trafficking of leukocytes into the skin, or a combination of both mechanisms. In vitro experiments demonstrate that BIX 642 and the anti-LFA-1 antibody effectively inhibit the production of IL-2 in squirrel monkey blood mononuclear cells stimulated by staphylococcal enterotoxin B. Therefore, efficacy in the delayed-type hypersensitivity model is likely to be a consequence, at least in part, of inhibiting costimulatory signaling. It is important to note that the 4-day dosing regimen was well tolerated with no overt signs of toxicity in these non-human primates.

BIX 642 was also effective in inhibiting the inflammatory reaction in the trans-vivo delayed-type hypersensitivity (Fig. 3). As found in the squirrel monkey delayed-type hypersensitivity, efficacy was comparable to that achieved with the anti-LFA-1 antibody. Both human (i.e., antigen presentation leading to T-cell activation) and mouse (i.e., trafficking of neutrophils) LFA-1-dependent events support the inflammatory reaction in the trans-vivo delayed-type hypersensitivity as evidenced by the efficacy of the anti-human and anti-mouse antibodies. Considering the selectivity of BIX 642 for human versus mouse LFA-1 (approximately 50-fold, P. Reilly, unpublished observations), the observed efficacy of this compound is likely a consequence of inhibiting human T-cell activation.

Our results demonstrate that these small molecule inhibitors effectively attenuate T-cell-dependent inflammatory reactions in vivo. The compounds inhibit both humoral and cellular effector stages of T-cell activation, most likely a consequence of blocking the costimulatory signaling of LFA-1/ICAM-1 interaction on T cells and antigen-presenting cells, respectively. Binding to LFA-1 may also inhibit T-cell activation by interfering with the formation of a functional immunological synapse (Grakoui et al., 1999).

Taken together, the results suggest that the BIRT analogues will be effective therapeutics in T-cell-mediated inflammatory diseases. These attributes are desirable when considering an effective new compound entity for autoimmune diseases such as psoriasis.

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