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ADVERSE REACTIONS TO SULFAMETHOXAZOLE IN HIV SEROPOSITIVE PATIENTS, IMMUNE ASPECTS

by

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ABSTRACT

Adverse reactions to sulfonamides were initially described more than 50 years ago. It is not yet clear whether these side effects are due to immunological reactions or drug toxicity. More than 50% of AIDS patients develop adverse reactions to sulfamethoxazole, a sulfonamide drug commonly used for the treatment of infections in these individuals. In order to study the possible role of the humoral immune response in adverse reactions to SMX in HIV-seropositive patients, I designed an ELISA to determine the classes, subclasses and the quantities of the immunoglobulins directed against SMX. Twenty eight HIV-seropositive individuals with a history of adverse reactions to SMX , 20 HIV-seropositive individuals with no such history, and 39 healthy infants and adults were evaluated for the presence of anti-SMX antibodies. Although most HIV-seropositive patients had antibodies to SMX, those with a history of adverse reaction to SMX had significantly higher levels of anti-SMX IgM and IgG antibodies. IgG_1 and particulary IgG_3 were the predominant subclasses of anti-SMX antibodies. The presence of high levels of IgG_3 suggests that complement may play a role in the adverse reactions to SMX in AIDS patients. Many individuals with low levels of anti-SMX antibodies had undergone treatment with this drug without side effects. Thus, it would appear that a low-level of antibodies to SMX can be tolerated without

overt symptoms. The specificity of the antibodies against SMX was analyzed by an inhibition assay, which demonstrated that SMX, SMX-poly-L-lysine (PLL), sulfisoxazole, sulfadiazine, N'-(2-thiazolyl) sulfanilamide, and N'-acetylsulfanilamide, all of which are structurally similar to SMX, could inhibit up to 80% of reactivity in the anti-SMX antibody assay. In contrast, no such inhibition was seen when less structurally related compounds such as TMP, sulfanilamide, 3-amino-5 methylisoxazole, and glycerol were used. These experiments suggest that the immunogenic determinant of SMX requires the presence of both sulfanilamide and 3-amino-5-methylisoxazole moieties in the same molecule.

To evaluate the cellular immune response in vitro, lymphocyte proliferation assays were performed in the presence of SMX or structurally related compounds. The lymphocytes from HIV-seropositive patients did not proliferate or produce antibody when incubated with these compounds. Only one immunocompetent individual who had a known hypersensitivity to SMX demonstrated high lymphocyte proliferation to SMX-PLL, but not to structurally related compounds.

The mechanism of the development of side effects to SMX is complex, and likely involves both toxic and immunologic events. It may be that high blood levels of SMX may lead to an immune response which produces symptoms only when specific antibody levels have exceeded a certain threshold. The detection of antibodies to SMX may be useful in predicting and

managing the adverse reactions to this compound in HIV-seropositive individuals.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ml:	Millilitre
IL:	Interleukin
Ig:	Immunoglobulin
MNC:	Monbnuclear cell
MW:	Molecular Weight
NT:	Not Tested
PBL:	Peripheral Blood Lymphocyte
PHA:	Phytohaemagglutinin
PWM:	Pokeweed Mitogen
ELISA:	Enzyme linked immunosorbant assay
AIDS:	Acquired Immunodeficiency Syndrome
FCS:	Fetal Calf Serum
°C:	Degree celsius
RT:	Room temperature
g:	Gram
hr:	Hour
min:	Minute
M:	Molar
nM:	Nanomolar
nm:	Nanometre
O.D.:	Optical Density
μg:	Microgram (10^{-6} gram)
μl:	Microlitre
DMSO:	dimethyl sulfoxide
PBS:	Phosphate Buffer Saline

PBS-T: Phosphate Buffer Saline and 0.5% Tween 20.

SMX: Sulfamethoxazole

TMP: Trimethoprim

PLL: Poly-L-Lysine

NACSN: N-acetyl-sulfanilamide

NTS: N'-(2-thiazolyl)sulfanilamide

3A5MI: 3-amino-5-methylisoxazole

SMX-PLL: sulfamethoxazole and Poly-L-Lysine

G-PLL: galactose and Poly-L-Lysine

SDZ: sulfadiazine

SNM: sulfanilamide

SFZ: sulfisoxazole

HIV+,+: HIV-seropositive patients with a history of adverse reaction to TMP-SMX.

HIV+,-: HIV-seropositive patients with no history of adverse reaction to TMP-SMX.

HLT-ADT: Healthy adults.

ALG: HIV seronegative, with a history of allergy to SMX.

N: normal

INTRODUCTION

Adverse reactions to sulfonamides were initially described more than 50 years ago. In 1940, Loveman and Simon reported cases of erythema nodosum after adminstration of sulfonilamide (De Weck and Bundgaard, 1983). After sulfonilamide was withdrawn from the market, a reduction in the cases of reported drug-induced erythema nodosum was observed (De Weck and Bundgaard, 1983), but reactions to sulfonamides remain quite common.

In the general population, 1-5% of patients show adverse reactions to these compounds. These side effects may be due to either immunological reactions (hypersensitivity) or drug toxicity. Sulfamethoxazole is the most commonly prescribed sulfonamide, often used in combination with trimethoprim as a preparation known as cotrimoxazole. This medication is the second most common cause of drug-induced rash (De Weck and Bundgaard, 1983; Anderson and Adkinson, 1987). Adverse reactions to cotrimoxazole are usually secondary to SMX (Gordin et. al.1984; Levy, 1989).

These adverse reactions can present as, skin eruptions (rash), febrile mucocutaneous syndromes (involving both the skin and mucous membranes), vasculitis, and hepatic hypersensitivity reactions (De Weck and Bundgaard, 1983; Anderson and Adkinson, 1987). The most severe reactions to sulfonamides are purpura and agranulocytosis which occur upon on the 10th to 12th day of high-dose therapy (Hartl ,1965, 1973; De Weck and Bundgaard, 1983; Victorino et. al., 1990).

Similarly, adverse reactions to SMX most frequently involve the skin, causing rash. But its administration may also result in fever, pancytopenia, and anaphylaxis (De Weck and Bundgaard, 1983; Gilman,1990). Most cases of adverse reactions to SMX occur 7 to 10 days after administration. Myositis, myocarditis, vasculitis with eosinophilia, Stevens-Johnson syndrome, systemic lupus erythematosus, and eosinophilic pulmonary infiltrates may also occur (De Weck and Bundgaard, 1983).

IMMUNE ASPECTS OF ADVERSE REACTIONS TO SULFONAMIDES, HAPTEN FORMATION

After administration to humans, sulfonamides undergo oxidation, reduction, hydrolysis, and conjugation, and may be transformed into protein-reactive *ortho-* or *para-* derivatives, resulting in the formation of immunogens (Erlanger et. al. 1973; Park et. al.,1978; Anderson and Adkinson, 1987; Uetrecht,1990). Protein binding of sulfonamides may be dose dependent. Fifty to 99% of SMX may bind to plasma proteins depending on the dose administered (De Weck and Bundgaard, 1983; Gilman,1990). These conjugates may then act as non-self immunogens and trigger an immune response.

Several strategies have been used to study the immune response to sulfonamides, including skin tests, the demonstration of sulfonamide-specific antibodies, or the

demonstration of sulfonamide-specific lymphocyte reactivity (Epstein et. al. 1990; Varga et. al.,1991). Experimental animal models and the study of clinical models have also provided some information on the pathogenesis of the adverse reactions to sulfonamides.

SKIN TESTS: Sulzberger et al (1947) and Cronin (1978) performed skin tests with sulfonamides in patients with serious reactions (De Weck and Bundgaard, 1983). One-third of patients showed positive skin tests. Using SMX- Poly-L-tyrosine, Gruchalla et. al (1991) also showed positive skin tests in such allergic patients.

SPECIFIC ANTIBODIES: Victorino et. al.,in 1990, showed the presence of leucocyte agglutinins in patients with agranulocytosis treated with sulfapyridine, SMX, or sulfadiazine. Hamilton and Sheets in 1978 demonstrated that thrombocytopenic purpura after sulfisoxazole therapy is antibody dependent (De Weck and Bundgaard, 1983). Antibodies reacting with SMX and TMP have also been described in patients developing adverse reactions (Ressler et. al.,1987,1990; Pears et. al.,1989; Epstein et. al. 1990 ; O'Neil et. al.,1991; Tatake et. al.,1991), but a correlation between levels of antibodies or IgG subclasses and the risk of adverse reactions has not been established. Anti-SMX antibodies have also been reported in HIV-seropositive patients who have a frequency of

adverse reactions to SMX of greater than 50% (Sullivan et. al.,1991; O'Neil et. al.,1991). Antibodies are of IgM , IgG, and in rare cases, IgE isotypes (Sullivan et. al.,1991; O'Neil et. al.,1991).

SPECIFIC LYMPHOCYTE REACTIVITY: The nature of the cell mediated response to sulfonamides is not clear. Han et al in 1969 showed blast transformation of the peripheral lymphocytes of a patient who developed a serum sickness-like disease due to sulfapyridine (De Weck and Bundgaard, 1983). However, since the assay was not specific for T-cells, this observation could have been due to B cell transformation and not due to T-cells. Other reports suggest that upon binding to a carrier, SMX is able to initiate blast transformation (Daniel et. al. ,1989; Epstein et. al. 1990), but the specific T-cell response to SMX is not well characterized. The assays to measure this response are not standardised and the results are difficult to interpret. SMX may also affect cell growth *in vitro*, which could additionally bias the assay (De Weck and Bundgaard,1983; Epstein et. al.,1990).

EXPERIMENTAL STUDIES IN ANIMALS: Aoki et al (1969) sensitized guinea pigs by repeated injections of p-hydroxylaminobenzene sulfonamide, a photo-oxidation product of sulfanilamide (De Weck and Bundgaard,1983).

Upon challenge with the drug an inflammatory reaction and

production of histamine were induced. Garrido et al (1979) demonstrated sulfonamide-specific IgE antibody in rats (De Weck and Bundgaard,1983).

CLINICAL STUDIES. Anaphylactic shock due to sulfonamides is a rare event (De Weck and Bundgaard, 1983). Antibodies to SMX have been described (Ressler et. al.,1987,1990; Sullivan et. al.,1991; Tatake et. al.,1991).

Increased complement activation has also been reported (O'Neil,1991). However, the correlation of the pathogenesis of adverse reactions to SMX with these antibodies and the involved subclasses is not clear. A serum sickness-like syndrome with fever, arthritic pains, urticaria, conjunctivitis, bronchospasm, and leukopenia has been associated with sulfonamide therapy (De Weck and Bundgaard, 1983; Honma et. al.,1988).

Some infections such as those caused by mycoplasma, herpes simplex viruses, haemolytic streptococci, salmonella, and Epstein-Barr viruses (EBV), may be co-factors of adverse reactions to sulfonamides (De Weck and Bundgaard, 1983). The activation of the immune system during an infection could predispose to adverse reaction to drugs. For instance, in primary EBV infections adverse reactions to ampicillin are frequent (Stites,1987; Uetrecht,1990).

TOXICITY TO SULFONAMIDES

The toxicity of sulfonamides is well recognized. Uetrecht (1990) suggested that sulfonamides are oxidized to protein reactive cytotoxic metabolites. Mononuclear leucocytes from patients with idiosyncratic reactions to sulfonamides were more susceptible to toxicity from these metabolites than were leucocytes from a control population, suggesting that these metabolites play a role in the pathogenesis of such reactions (Cribb et. al.,1990,1991; Epstein et. al. 1990). SMX is converted to a reactive and electrophilic component by means of enzymatic activity of immunocompetent cells (Uetrecht, 1990; Van Der Van der,1991). Oxidation of sulfamethoxazole at the N4-position forms a hydroxylamine, which may be toxic to lymphocytes (Van Der Van der,1991). The formation of the hydroxylamine is dependent on the presence of microsomes, NADPH, and oxygen (Cribb,1990).

Patients treated with long acting and ultra-long-acting sulfonamides could have progressive accumulation of the drugs by increased protein binding, and may be prone to severe drug reactions, such as severe allergic dermatoses or erythema multiforme gravis on this basis (De Weck and Bundgaard, 1983). Administration of high doses of TMP-SMX may induce suppression of the bone marrow, which could result in neutropenia. This toxicity may be due to both SMX and TMP (De Weck and Bundgaard, 1983; Victorino et. al., 1990; Cribb et. al.,1990, 1991; Van Der Vans, 1991). Sulfonamides administered in high

doses or for long periods of time may decrease myelocytopoeisis through a selective inhibition of DNA synthesis (De Weck and Bundgaard, 1983). In certain microorganisms, TMP inhibits the production of folate, but, mammalian cells are not dependent on this pathways, deriving this compound from dietary sources (Gilman,1990). There is no evidence for any effect of TMP on mammalian protein synthesis. However, a direct suppressive effect of TMP on stem cells is observed. Bone marrow suppression often follows treatment with high dose TMP, and this may be controlled by the administration of exogenous folate. However, some authors have failed to show any beneficial effect of folate supplements (Bygbjerg,1988; Levy,1989; Gilman,1990).

IMMUNE REACTIONS INVOLVED IN ADVERSE DRUG REACTIONS

The Gell and Coombs classification of hypersensitivities is based on the pathogenesis of immune reactions to drugs (Gell and Coombs, 1964; Roitt,1988), which can be type I, II, III, and IV.

Type I hypersensitivity:

Type I hypersensitivity is mediated by antibodies of the IgE isotype. Mast cells or mastocytes have Fc receptors for IgE. After the first exposure to an allergen, allergen-specific IgE is synthesized and mast cells become sensitized. A second exposure to the same allergen leads to mast cell

degranulation, as a result of cross linking of at least two bound IgE molecules on the surface of mast cells. The consequence of this degranulation is the release of different mediators such as histamine and many other potent biological compounds. This follows the activation of adenyl cyclase and methyl transferase, causing production of protein kinase through increasing cAMP and intracellular calcium and activation of phospholipase A2, resulting in intermediate filament formation. The consequences of these reactions are the movements of the granules to the cell surface and their release. At the same time release of arachidonic acid activates lipoxygenase and cyclooxygenase pathways and produces mediators such as leukotrienes and prostaglandins, respectively (Stites,1987; Roitt; 1988; Tizard,1988). This type of hypersensitivity is sometimes divided into two phases, an immediate phase which may start in about ten minutes, and a late phase which begins later (in about 12 hours) following production of leukotrienes.

Upon binding to carriers some drugs may induce IgE production. For example, penicilloyl is an active derivative of penicillin and is able to bind to proteins. When the drug is administrated parenterally the reactions may occur immediately, whereas following oral adminstration of the drug, reactions may take up to six hours to occur (Stites,1987; Roitt; 1988; Tizard,1988). A wide range of compounds may cause IgE-mediated hypersensitivity (antibiotic, foreign

antisera, other blood products, chymopapain and asparaginase, polypeptide hormones, vaccines such as tetanus toxoid, influenza, and other egg-containing vaccines, iron dextran) (Stite,1987; Roitt; 1988; Tizard, 1988).

Sulfonamides, local anaesthetics and salycilates also have been reported to initiate IgE mediated reactions in humans. Anaphylactic shock upon receiving TMP-SMX is rare, with most reported cases being attributed to hypersensitivity to TMP (Harle and Baldo,1987; Arnold et. al.,1988; Pears and Morley, 1989). However, IgE antibodies to SMX have also been described (Sullivan et. al.,1991).

Type II hypersensitivity or Cytotoxic reactions

Type II hypersensitivity is associated with the destruction of cells by means of antibodies and/ or complement. Cytopenias have been frequently associated with the adminstration of drugs (Worlledge,1973; De Weck and Bundgaard,1983; Anderson and Adkinson,1987; Uetrecht, 1990). Drugs may bind to a cell surface and antibodies are directed against either the drug or the cell surface antigen. Alternatively, upon production of specific antibodies, drugs may act as haptens and bind to these antibodies and the resulting immune complexes may bind to the receptors for the Fc portion of the IgG on the surface of red blood cells (De Weck and Bundgaard,1983; Anderson and Adkinson,1987; Uetrecht, 1990).

Additionally, if a drug interacts with a cellular HLA antigen, this complex may be recognized by a T-cell, resulting in a humoral or cellular response against a self-antigen (Roitt,1988). Thus, by this mechanism, a breakdown of tolerance would occur, leading to autoimmune disease (Erlanger et. al. 1973; Anderson and Adkinson, 1987; Roitt, 1988; Bell,1989; Uetrecht,1990; Kenna,1988). In this way, thrombocytopenia may follow therapy with quinine, quinidine, acetaminophen, thiouracil, gold salts, and sulfonamides (Anderson and Adkinson, 1987; Sautois et. al.,1991).

Treatment of patients with TMP-SMX has also been frequently associated with pancytopenia and with antibodies against granulocytes and platelets (Weinke et. al.,1988; Pygbjerg,1988; Levy,1989; Jeurissen et. al.,1989; Finegold et. al.,1986; Victorino et. al.,in 1990; Sullivan et. al.,1990). In most of these cases antibodies against SMX were the cause of the immune reactions.

Type III hypersensitivity or Immune-complex reactions

Drugs that are immunogenic may form immune-complexes in the presence of specific antibodies (Anderson and Adkinson, 1987; Roitt, 1988; Ten et. al. 1988, Singer et. al.,1988; Rudra et. al., 1989). Soluble immune complexes along with complement are able to initiate type III hypersensitivity reactions. Serum sickness is a systemic type III hypersensitivity reaction characterized by fever and rash.

Examples of drugs associated with type III reactions are: penicillin, sulfonamides, thiouracil, cholecystographic dyes, hydantions, aminosalicylic acid, and streptomycin (Anderson and Adkinson, 1987).

Adverse reactions to SMX have been shown to be associated with decreased C3 and C4, which suggests immune complex formation (Anderson and Adkinson, 1987; Alberti-Flor et.al., 1989).

Type IV hypersensitivity or Delayed type hypersensitivity

Type IV hypersensitivity is caused by the interaction of processed antigens and T cells (Roitt, 1988). In mice T cells involved in DTH reactions belong to the T-helper 1 class or Th1. Hence, they predominantly secrete IL-2 and gamma interferon (Th2 cells predominantly secrete IL-10, IL-4, and IL-5) (Benjamin et. al., 1992).

There are many clinical manifestations of adverse drugs reactions that seem to be related to T cell activation, however only a few cases have been confirmed to be T cell mediated (De Weck and Bundgaard, 1983; Anderson and Adkinson, 1987; Brattig, 1988; Aguilar Mezquita, 1989; Baldo,1990; Kimber et. al.,1990). Neomycin, a topical antibiotic, frequently causes contact dermatitis, which is known to be mediated by DTH (Benness, 1992).

The administration of nitrofurantoin, penicillin, or hydantoin is also associated with type IV hypersensitivity

reactions (De Weck and Bundgaard, 1983; Anderson and Adkinson, 1987; Ten et. al. 1988, Singer et. al.,1988). To date, no clear association of sulfonamide adminstration with such reactions has been described.

Complex adverse drug reactions may have components from different types of hypersensitivity, and may not fit into a single category of the Gell and Coomb's classification. However, it remains a good model for the study of such reactions, especially if we are seeking to increase our understanding of the mechanisms involved.

HIV INFECTION AND SMX THERAPY

Opportunistic infections are common in the late stages of HIV infection (Levy,1989). Cotrimoxazole is widely used for the treatment of a variety of bacterial and parasitic infections including Pneumocystis carinii pneumonia (PCP), the most frequent and severe opportunistic infection in HIV-seropositive patients (Levy, 1989; Van Der Ven, 1991). Without therapy Pneumocystis carinii pneumonia in AIDS patients is almost universally fatal. The major alternative therapy, pentamidine, is highly toxic and about half of the patients cannot complete a full course of therapy. It causes azotemia, liver function abnormalities, hypoglycaemia, hypotension, rash, and hypocalcemia(Levy, 1989; Van Der Ven et. al.,1991,1992).

TMP-SMX ADVERSE REACTIONS IN HIV INFECTION

The use of cotrimoxazole in the treatment of PCP infection is hampered by the high frequency of adverse drug reactions in AIDS patients. In the general population, only 1-5% of patients exhibit adverse drug reactions to TMP-SMX, but in HIV-seropositive patients 40% to 80% may develop such reactions (Gordin et.,al.,1984; Lee et. al.,1987; Weinke et. al., 1988; Levy,1989; Van Der Ven et. al.,1991,1992). In addition to frequent nausea and gastrointestinal intolerance, common reactions among AIDS patients include rash, leukopenia, and thrombocytopenia (Levy,1989).

These reactions generally begin 8-10 days after therapy is initiated. However, there are several reports showing anaphylactic reactions at the time of the first dose (Van Der Ven et. al., 1991,1992). These reactions may be either due to TMP or SMX. In advanced AIDS, IgE responses to SMX may develop (O'Neil et. al.,1991).

The blood levels of cotrimoxazole may be important in the development of adverse reactions especially in HIV-seropositive patients. TMP reaches levels above 5 *ug*/ml while the level of SMX may exceed 100 *ug*/ml (Lee et. al.,1987; Levy, 1989). Sulfadiazine is structurally similar to SMX. Therapy with high-dose sulfadiazine, has also shown to be associated with a high prevalence of adverse reactions among AIDS patients (Caballer de la Hoz,1991).

POLYCLONAL ACTIVATION OF B-CELLS IN HIV INFECTIONS

Increased levels of total IgG and IgE and SMX-specific IgE have been described in advanced AIDS (Wright et. al., 1990; O'Neil et. al.,1991). It is possible that in the late stages of AIDS, Th2 cells are the predominant subtype of T lymphocytes, which could cause an excessive B-cell and humoral responses. Th2 cells are known to product IL-4, IL-5, and IL-10. Benjamin et al (1992) have recently shown the increased secretion of IL-10 in cells from AIDS patients.

The polyclonal activation of B-cells seen in HIV infection may be due to several factors, including the onset of multiple infections that elicit a strong humoral immune response, the reduction of suppressor T-cells activity, EBV transformation of B-cells. An autoimmune reactivity may also contribute induction of polyclonal activation of B-cells.

The polyclonal activation of B-cells and the high blood levels of SMX may act synergistically to produce an exaggerated humoral response to SMX in AIDS patients.

RATIONAL AND OBJECTIVES

A number of factors suggest that immunological mechanisms may account for the adverse drug reactions to SMX in immunocompetent patients: a) anaphylactic reactions (Arnold et.,al.,1988); b) immune-complex formation (Alberti-Flor et. al.,1989); c) positive skin tests with the drugs or its conjugates; d) time course of reactions (Gruchalla et. al. 1991); e) specific antibody to the drug; and f) reactions upon readminstration of the drug (Ressler,1987).

A number of findings also suggest that immunological mechanisms may play an important role in similar reactions in HIV-seropositive individuals. a) Anti-SMX antibodies have been identified in some patients (O'Neil et. al.,1991; Sullivan et. al.,1991). b) Desensitization may allow for successful adminstration of the drug (Finegold et. al.,1986; Papakonstantinou et. al. 1989; White et. al. 1989). c) Steroids are useful in the treatment of reactions (Gruchalla et.al., 1991; O'Neil et. al.,1991).

Based on previous investigations on the pathogenesis of adverse reaction to SMX, I propose that the humoral response to SMX is involved in the pathogenesis of the adverse reactions seen in HIV-seropositive patients, and that patients with high levels of specific IgG or IgM are at increased risk of developing such reactions.

With this in mind, the aims of this study were to evaluate the humoral and cellular responses to SMX. In order to achieve these aims, I had the following objectives:

1- To design an ELISA to measure the classes of antibodies to SMX, and to determine IgG subclasses reacting with SMX.

2- To measure the humoral and cellular responses to SMX in vitro.

MATERIALS AND METHODS

Patient Population, sera and lymphocytes

The patient population in this study consisted of HIV-seropositive individuals with a history of an adverse reaction to SMX. The control population included HIV-seropositive patients, and normal adults and infants with no history of such reactions, and one HIV seronegative patient with a known allergic reaction to SMX. This study was approved by the Human Experimentation Committee at the Ottawa General Hospital, University of Ottawa.

Informed consent was obtained from all study subjects prior to their participation. Single serum samples were obtained from 40 HIV-seropositive individuals, 20 with a history of adverse reactions following 5-10 days of therapy with TMP-SMX, and 20 individuals who had taken sulfonamides in the past with no such reactions. Sera were kept in -20 C° until the day of experiments. The control population consisted of 20 infants (age, 12 ± 3 months), and 18 healthy adults with no history of adverse reactions to TMP-SMX. The infants were chosen from patients admitted to hospital because of bronchiolitis, bronchitis or fever and rash. The healthy adults were laboratory workers. None of the infants or healthy adults had received SMX. Thus, 20 HIV-infected individuals with a history of adverse reactions to TMP-SMX and 58 subjects with no such history were studied (20 HIV-seropositive, 20 infants, and 18 healthy adults).

For the study of the cellular response to SMX, twelve HIV- seropositive patients, six of which had a history of adverse reaction to SMX, six HIV-seronegative healthy individuals, and a patient with a known allergic reaction to TMP-SMX were selected. Heparinized fresh blood was drawn from all studied subjects and isolated mononuclear cells were used in a lymphocyte proliferation assay. Blood was diluted (1:3) in RPMI. Ten ml diluted blood was then gently layered on 5 ml of Ficoll-Hypaque, taking care not to disturb the interface. After a 25 min. centrifugation at 400 g. at room temperature, the plasma-Ficoll interface (accumulated, viable, mononuclear cells) was collected. The cells were resuspended in PBS, then centrifuged for ten minutes at 400 g, at room temperature. The supernatant was discarded and the pellet was resuspended in RPMI and centrifuged at 250 g for 5 minutes at room temperature. The pellet was resuspended in 2 ml RPMI and the number of cells was counted in a haemocytometer. These cells were then used for the evaluation of the cellular response to SMX.

Supplies

Polystyrene flat-bottom 96-well Polysorb microtitre plates were obtained from NUNC (NUNC, Gibco, Montreal,Canada). Washing was done with a SLT-Labinstruments Multiwash automatic plate washer and with PBS containing 0.5% Tween 20. Tween 20 was purchased from Sigma (St Louis,MO, USA). Plates were read

using a Dynatech MR600 plate reader. Sulfamethoxazole and trimethoprim, sulfisoxazole, sulfanilamide, sulfadiazine, 3-amino-5 methylisoxazole, N'-(2-thiazolyl) sulfanilamide, N'-acetylsulfanilamide, sodium salt were purchased from Aldrich (St. Louis,MO,USA), and Poly-L-lysine, BSA fraction V(7.5), phosphate-citrate buffers, glycine, and OPD were purchased from Sigma (St Louis,MO,USA). Horseradish Peroxidase conjugated anti-IgM and anti-IgG were purchased from TAGO Inc. (Burlingame, CA,USA). Horseradish Peroxidase conjugated anti-IgE and subclasses of IgG were obtained from Active Site (Birmingham, England). Glutaraldehyde was purchased from JBS Supplies (J.B. EM Services Inc. Montreal,Canada).

^{3}H-Thymidine was obtained from New England Nuclear (Boston, MA, USA). A liquid scintillation counter, 1214 RACKBETA (LKB,Sweden) Wallac and a semiautomatic cell harvester, Skartron and Filtermats (Skatron inc. Sterling, VA,USA) were also used. CellTiter 96 NON-Radioactive Cell Proliferation/ Cytotoxicity Assay was purchased from Progema Corporation (WI, USA).

BSA, Iscove's modified Dulbecco's medium and Phosphate-Buffered Saline (PBS) were obtained from Sigma (St Louis,MO, USA). Sterile 100 ml bottles of Ficoll-Hypaque were purchased from Pharmacia (Piscataway, NJ,USA) and stored at 4°C. A 3.0 M NaOH from Sigma served as the ELISA's stop solution. A refrigerated bench top centrifuge, Model TJ-6 Refrigerated Centrifuge, was purchased from Beckman Instruments Inc.

(Fullerton, CA,USA). Cell cultures were maintained in an automatic CO_2 incubator purchased from Precision Instruments Inc.(Rockford,IL,USA). Throughout the cell incubation periods this incubator was maintained at a temperature of 37°C ± 0.5 and 5.0 ±1% CO_2 . Inverted phase microscopes and regular light microscopes from Zeiss (West Germany) as well as a Corning pH metre (model 40) from Fisher Scientific Co. (Pittsburgh, PA,USA) were also used.

HUMORAL RESPONSE TO SMX

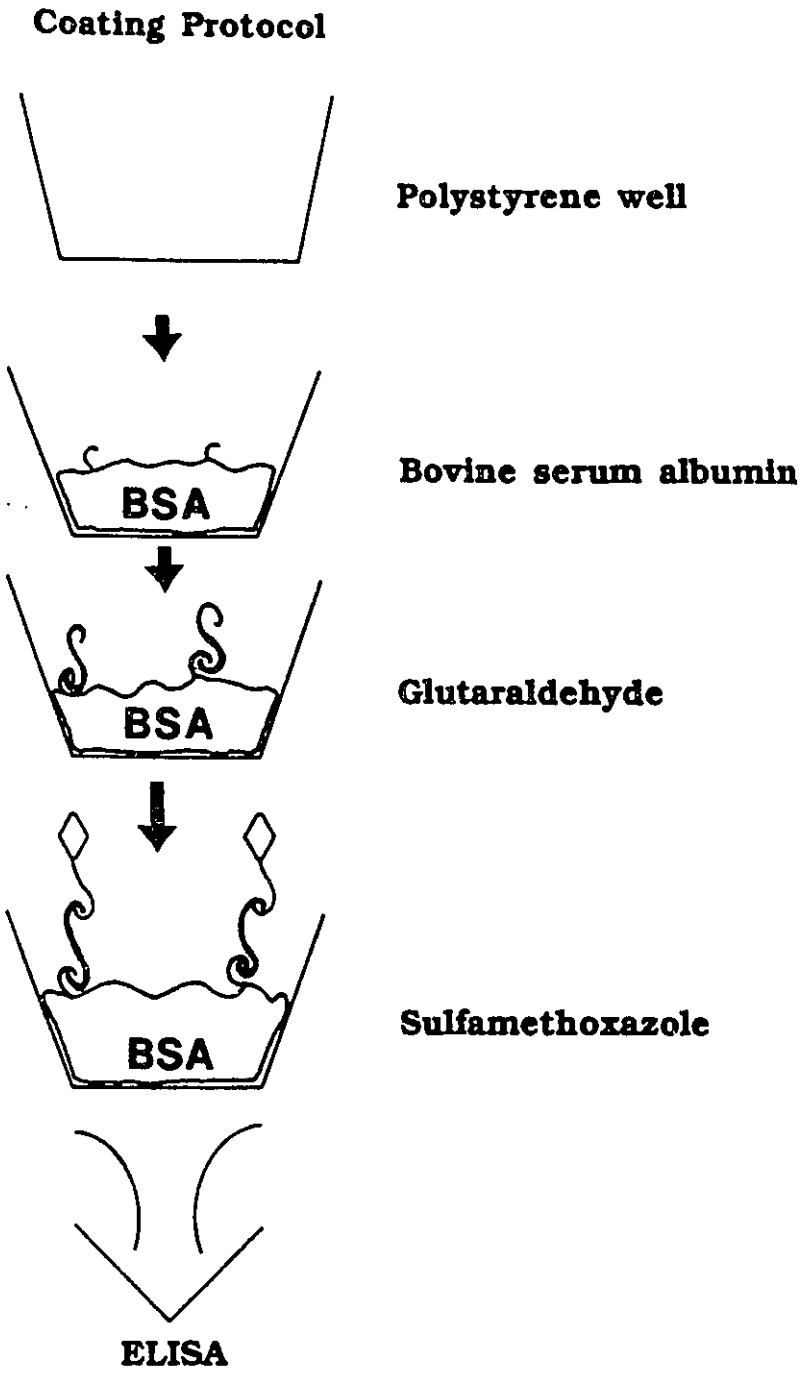
To study the humoral response to SMX, an ELISA method to measure antibodies to SMX was designed. To obtain an antibody capture ELISA, the antigen is attached to the bottom of a polystyrene tube or plate. However, having a molecular weight of 253 dalton, SMX is a hapten which does not bind to polystyrene by itself. Therefore, SMX was conjugated to a carrier, that was coated in the polystyrene plates. This carrier, thus, acted as a linker. On the other hand, in body liquids, upon adminstration, SMX is partly metabolised to electrophilic derivatives such as hydroxylamine-SMX which are able to interact with proteins (95% with human serum albumin) (Gilman et al, 1990). Using carriers with the same size of human serum albumin could enable us to mimic in vivo interactions of SMX.

PREPARATION OF ANTIGENS FOR ELISA

A common carrier used for making a hapten attachable to polystyrene plates is BSA. However, althou it has some structural similarity with human serum albumin, a possible exposure of this antigen to human may lead to high background readings in the ELISA. Therefore, poly-L-Lysine, which has also been used as a carrier, was also employed. Human sera are not supposed to have antibodies against poly-L-Lysine, thus the background of ELISA could be less than that of a BSA based assay.

These two separate strategies were pursued in parallel for the preparation of antigens which were then used in the ELISAs for the detection of anti-SMX antibodies. In the first approach, SMX was conjugated to bovine serum albumin (BSA) using glutaraldehyde. Polystyrene flat-bottom 96-well microtiter plates were coated with BSA-glutaraldehyde as described by Saunders (Saunders et. al.,1973). Three μg of BSA in 0.1 ml of distilled water was added to each well and allowed to dry at 37°C (1 hr), after which 0.1 ml of 0.2% glutaraldehyde (pH 7.2) was added (Figure 1). The plates were incubated for 30 min at 37°C, then washed 5 times with distilled water. One μg of SMX in PBS (pH 8.0) was added to each well, and the plates were incubated for an additional two hours at room temperature. This was followed by the addition of 200 μl of 1M glycine (to saturate any remaining glutaraldehyde) buffer in PBS (pH 7.2) to each well and a

Figure 1. Preparation of BSA-glutaraldehyde-SMX conjugate and its use to coat the ELISA plate. Conjugation and coating steps were performed at the same time.


Coating Protocol
Polystyrene well
BSA
Bovine serum albumin
BSA
Glutaraldehyde
BSA
Sulfamethoxazole
ELISA

further 1 hr incubation at 37°C. Finally, the plates were washed 5 times with PBS-T and blocked with 1% gelatin in PBS, pH 7.2, for 2 hr at room temperature and stored at 4°C for future use.

The sera of nine HIV-seropositive patients with a history of reactions to TMP-SMX and eight HIV-seronegative patients with no history of reactions to TMP-SMX (controls) were tested using this plate bond conjugate.

In the second approach , SMX was conjugated to poly-L-lysine, this approach was completely performed in Dr. R. Roy's lab, department of chemistry, the University of Ottawa. The free amine group of SMX was N-acryloylated with acryloyl chloride in methanol/triethyl amine as described previously for carbohydrate haptens (Roy et. al.,1991,1988, 1988). The pure acryloylated SMX was recovered with a 76% yield. The N-acrylamido SMX derivative was covalently coupled to poly-L-lysine by a Michael addition of the free ϵ-amine function of the lysine residues. The conjugation was performed in carbonate buffer (0.2 M, pH 10) containing dimethyl sulfoxide (DMSO) (9:1) at room temperature for 4 days. Dialysis (1 mM HCl), followed by lyophilization gave the SMX-poly-L-lysine conjugate as a white, spongy solid. The product was purified and fully characterized by elemental analysis, ^{1}H and ^{13}C-NMR spectroscopy, mass spectrometry and U.V. spectroscopy. ^{1}H-NMR analysis indicated that 33% of the lysine residues in the new conjugate had been modified with the N-acryloylated SMX. Co-

polymerization of SMX with acrylamide in 4/1 H_2O/SMSO solvent system by radical initiation with $(NH_4)_2$ S_2O (1.25 mg) at 100°C for 45 minutes gave 148.3 mg (90% yield) of the desired copolymer. The polymer was dialysed against multiple 5:1 volumes of H_2O to remove low molecular weight species then freeze-dried.

Elemental analysis and ^{1}H-NMR indicated a 1:12.4 SMX: acrylamide incorporation ratio. Elemental analysis also showed 13.6% of retained water in the lyophilized material.

Twenty HIV-infected individuals with a history of adverse reactions to TMP-SMX, 20 HIV-infected individuals with no such history, 10 healthy adults, and 20 infants were studied using using this plate-bond conjugate.

ELISA PROTOCOL- STANDARDIZATION OF REAGENTS

Having conjugated the hapten (SMX) with carriers, I was able to perform an ELISA for anti-SMX antibodies as a method to evaluate the humoral response to SMX.

To determine the concentration at which the conjugates gave the best result in coating, different concentrations of SMX-BSA and SMX-PLL were used with certain sera.

To estimate the best reacting dilution of any anti-Ig vials with the least background, every vial of anti-Ig antisera to subclasses of IgG, IgM, and IgE were diluted and evaluated using the ELISA protocol with the same serum. The optimal dilution were for anti-IgM (1/3000), for anti-IgG

(1/1500), for anti-IgE (1/6000), for anti-IgG_1 (1/160), for anti-IgG_2 (1/160), for anti-IgG_3 (1/160), and for anti-IgG_4 (1/160).

Plates were coated with 1μg of SMX-PLL in 100 μl of PBS for each well and were incubated in 4° C overnight. 100 μl of serum diluted 1:40 (this was determined empirically, range of dilutions from 1:40 to 1:100 were chosen based on a lower background) in PBS (pH 7.2) was added to each well. After 1-2 hr incubation at 37°C the plates were washed 3 times with PBS-T (PBS and 0.5% Tween 20). 100 μl of appropriate dilutions of peroxidase conjugated anti-IgM (1/3000), anti-IgG (1/1500), anti-IgE (1/6000), anti-IgG_1 (1/160), anti-IgG_2 (1/160), anti-IgG_3 (1/160), and anti-IgG_4 (1/160) were added. After a 1 hr incubation at 37°C, the plates were washed 3 times in PBS-T and the O-phenylenediamine dihydrochoride (OPD) substrate was added to each well. After a 40 minute incubation at room temperature colour development was measured as OD at 450 nm with an automated plate reader (One normal H_2SO_4 was used to stop the reactions).

Hapten Inhibition Assay:

A hapten inhibition assay is required to study the specificity of a given ELISA protocol for a particular hapten. Therefore, before running the ELISA, different compounds, structurally similar to SMX, are preincubated with the test sera. Thus, these compounds will be assessed for their

ability for interaction with anti-SMX antibodies. On the other hand, since SMX is mainly prescribed in combination with trimethoprim (TMP), therefore, TMP was also used to know if anti-SMX antibodies can interact with it.

Sulfisoxazole, sulfadiazine, N'-(2-thiazolyl) sulfanilamide, 3-amino-5-methylisoxazole and N'-acetyl sulfanilamide, all of which are structurally similar to SMX, as well as TMP have been used in this hapten inhibition assay.

Fifty μl sera of patients and controls diluted (1/40 - 1/100) were incubated (RT) for 1 hr with 50 μl of solutions of the PLL, TMP, SMX, SMX-PLL, sulfisoxazole, sulfanilamide, sulfadiazine, 3-amino-5-methylisoxazole, N'-(2-thiazolyl) sulfanilamide, N'-acetylsulfanilamide, sodium salt all in PBS in the concentration of 1 nM to 1000 nM (pH 7.2). The ELISA was then performed as described above.

Anti-SMX antibodies in sera were measured by the ELISA without addition of any inhibitor. Inhibitors were added and after a 2 hr. incubation, the OD was again determined in a separate Elisa assay. ODs in the absence of inhibitor (I_o) and ODs in the presence of inhibitors (I_c) were used in the acquisition to calculate the per cent of inhibition:

$$\text{Per cent of inhibition} = \frac{I_o - I_c}{I_o} \times 100$$

CELLULAR RESPONSES TO SMX

Immune response of any kinds, humoral or cellular,

requires "help" from T cells. Some clinical evidences of SMX adverse reactions, also suggested a type IV hypersensitivity reaction to be involved (in which T cells are involved).

^{3}H-Thymidine incorporation assay

To study the cellular response of patients and controls in vitro, one can incubate isolated mononuclear cells with the antigen and evaluate the proliferation of these cells by different means. ^{3}H-Thymidine incorporation assay is one of methods used for this evaluation. In this assay, ^{3}H-Thymidine is added to the plates in the last hours of incubation, so that it is incorporated in the proliferating cells. Radioactivity is measured employing a liquid scintillation counter.

^{3}H-Thymidine incorporation assay was done for twelve HIV-seropositive, six of which with a history of adverse reaction to SMX, six HIV seronegative healthy individuals, and a patient with a known allergic reaction to TMP-SMX.

Heparinized (50 units heparin/ml of blood), fresh blood of patients and controls were used to isolate lymphocytes by means of Ficoll-Hypaque. Different concentrations (0.01 μg, 0.10 μg, 1.00 μg ,10.00 μg all in 100μl) of SMX, SMX-PLL, PLL, sulfanilamide, TMP, and Galactose-PLL were incubated with 500 000 cells of patients and controls. Wells with 100 μl of RPMI and 10%FCS as a negative control and with PWM (1% in media) as a positive control were also incubated with all cells. This

was followed by 5-7 days incubation at 37°c with 5% CO_2. The cells were then incubated with 1μ Ci ^{3}H-thymidine for 16 hours (Ci was diluted in media in a final volume of 25 μl was added to each well). This incubation, was followed by harvesting cells. Harvested cells were transferred into appropriate labelled tubes and 2ml of solubilizing solution (Ready Proten) was added to each tube. The radioactivity of the cell culture was determined in a liquid scintillation counter.

CellTiter 96 Non-Radioactive Cell Proliferation assay

CellTiter 96 Non-Radioactive Cell Proliferation assay is an alternative method for determining viable cells and cell proliferation (Mosman, 1983). This assay is based on the cellular alteration of a tetrazolium salt into a blue formazan yield which is detected by ELISA reader.

Twelve HIV-seropositive patients, six of whom with a history of adverse reaction to TMP-SMX, six HIV-seronegative healthy individuals and one patient with a known allergic reaction to TMP-SMX were studied. In different assays, 1000000, and 500000 mononuclear cells of these patients and controls were incubated with different concentrations (0.01 μg, 0.10 μg, 1.00 μg, 5.00 μg, 10.00 μg all in 100 μl RPMI-FCS) of SMX, SMX-Poly-L-lysine (PLL), PLL, sulfanilamide, TMP, RPMI-FCS, PWM, and Galactose-PLL (the concentration of PWM is 1/100 in RPMI-FCS). This was followed by 5-7 days incubation in 37°C with 5% CO_2. After incubation the dye was added (15

μl tetrazoliom dye for each 100 μl) and plates were left in humid for four hours. This was followed by the addition of 100 μl of the solubilization solution to each well. The absorbency was then determined at 570 nm against reference of 630 nm.

Measurement of immunoglobulins in supernatant

In order to study the presence of Ig and anti-SMX antibodies in the supernatant of these cell assays, ELISA techniques were employed. In an antibody capture test, the plates are initially coated with anti-IgG,M,A antibodies to capture any antibody of the same class in the samples. Anti-IgG,M,A conjugated with horse radish peroxidase are added to the wells. Finally, a colour will develop upon addition of substrate. At each step, the all traces of unbound reagents have to be removed.

Goat-anti-Human-IgG,A,M in appropriate dilutions (1μg per well, determined by preparing a dilution and running the test with standard) were added to Nunc polysorb plates. Plates were sealed with sealers (Myler plate sealers,ICN-Biomedicals) and placed in a humid place, for at least 18 hours at room temperature, after which the plates were stored at 4°C. The plates were washed with PBS and 0.5% Tween 20 (PBS-T), for 4 times. The serum of a male healthy adult was diluted (1/10,1/20, 1/40, 1/80, 1/160,1/320,and 1/640) with PBS-T to be used as an standard. 100 μl of diluted serum and

media as well as 100 μl of each culture well supernatant were added to the appropriate well of the plates. The plates were then incubated at 37°C for 1 hour, then washed for 4 times with PBS-T. This was followed by the addition of 100 μl of peroxidase conjugated anti-human-IgG,A,M, in appropriate dilutions, to each well (1/3000 for IgG,1/1500 for IgA, and 1/4000 for IgM, determined by preparing a dilution and running the test with certain samples). After another incubation at 37°C for 1 more hour, the plates were washed 4 times as above. Substrate was prepared just before use. Tablets of O-phenylenediamine dichloride were dissolved in citrate phosphate buffer and 30% H_2O_2 (i.e. 4 mg OPD, 10 ml citrate phosphate buffer, and 4μl H_2O_2). 100 μl of substrate was added to each well, followed by stopping the reaction with 100 μl of 1 normal H_2SO_4 and a 30 min incubation in dark. Plates were read with a MR 600 Microplate Reader, at a test wavelength of 450 nm and a reference wavelength of 630 nm.

RESULTS

RESULTS OF HUMORAL RESPONSE TO SMX

Interpretation of the ELISA results

ELISA's OD values above two standard deviations above the mean OD measurement of the infant's and adult volunteers was used as criteria to define the presence of antibodies to SMX in patient's sera. Infant's sera were chosen as negative controls because they were less likely to have received TMP-SMX. All infants were between 12 ± 3 months of age, and were unlikely to have detectable levels of maternal antibodies.

Optimization of Conjugate Concentrations

In order to find the optimal concentration of conjugates, dilutions of SMX-BSA or SMX-PLL from 1 to 10 μg (per well) were bound to the solid phase after overnight incubation at 4°C, using aqueous diluent at neutral or alkaline pH (PBS). The best concentration of SMX-BSA was 3 μg of 100 μl of PBS and of 1μg of SMX-PLL in 100 μl of PBS in each well (Table 1).

ELISA with SMX-BSA conjugate

In the first approach of conjugation, ELISA was performed on the sera of nine HIV-seropositive patients with a history of reactions to TMP-SMX and in eight HIV-seronegative patients with no history of reactions to TMP-SMX (controls).

Table 1. Anti-SMX antibodies of positive and negative controls upon coating of plates with different concentrations (1 to 10 μg/ml) of SMX-BSA and SMX-PLL conjugates. The best results were obtained with 3 μg/well of SMX-BSA and 1 μg/well of SMX-PLL. +ve: ODs of ELISAs using sera of the 4 HIV-seropositive patients with adverse reactions to SMX.
-ve: ODs of ELISA using of the sera of 4 healthy adults with no history of adverse reactions to SMX.

conjugate	SMX-BSA		SMX-PLL	
µg/well	+ve	-ve	+ve	-ve
1.00	1.30	0.34	0.95	0.18
2.00	1.42	0.35	0.83	0.19
3.00	1.52	0.35	0.87	0.18
4.00	1.50	0.40	0.86	0.20
5.00	1.47	0.39	0.81	0.21
6.00	1.41	0.38	0.80	0.20
7.00	1.38	0.38	0.81	0.20
8.00	1.35	0.38	0.79	0.18
9.00	1.35	0.39	0.83	0.21
10.00	1.34	0.38	0.77	0.24

The results of the ELISA using the SMX-glutaraldehyde-BSA conjugate for 9 patients and 8 controls are summarized in Table 2. IgG and IgM specific to SMX were elevated in HIV-seropositive patients when compared to healthy adults ($P<0.01$).

The ODs of IgG results of patients were approximately three times more than that of controls (Table 2). Whereas, the IgM results were about 4.5 times higher than that of controls and it seems that probably IgM plays an essential role in these adverse reactions (Table 2). Thus, SMX specific IgM may be a key in the diagnosis of adverse reactions to SMX.

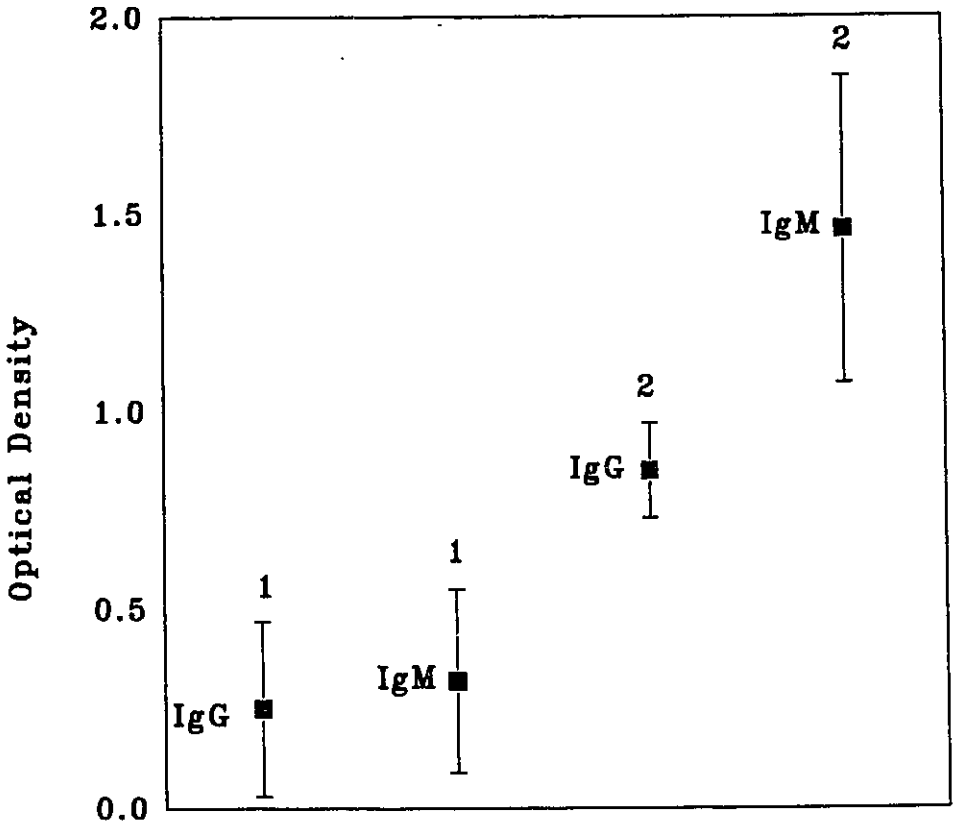
The distribution of ODs of this experiments is shown in Figure 2. Using SMX-glutaraldehyde-BSA in the ELISA a remarkable background, particulary for the IgM assay, was noticed (an OD of approximately 0.30). This background could be due to cross-reacting antibodies, and/or presence of antibodies to BSA in the sera. Changing the carrier would allow us to determine if the background was due to BSA.

Evaluation of the IgG subclasses reacting with SMX has not been reported so far. To measure IgG subclasses, an alternative method of conjugation was used to avoid high background ODs and to improve sensitivity.

Table 2. ELISA with BSA-glutaraldehyde-SMX conjugate for the detection of anti-SMX IgG and IgM. ELISAs were performed with the BSA-glutaraldehyde-SMX conjugate method for the detection of anti-SMX IgG and IgM in nine HIV-seropositive patients with a history of reactions to TMP-SMX and in eight HIV-seronegative patients with no history of reactions to TMP-SMX (controls). ODs of IgG and IgM assays patients' sera were significantly ($P<0.01$) higher than that of controls.

Immunoglobulin classes	Patients (n=9)	Controls (n=8)	P value
IgG (X ± SD)	0.85 ± 0.12	0.25 ± 0.22	<0.01
IgM (X ± SD)	1.46 ± 0.39	0.32 ± 0.23	<0.01

Figure 2. Distribution of ODs of anti-SMX IgM, and IgG tests and controls in the ELISA with BSA-SMX (glutaraldehyde method). Anti-SMX IgM, and IgG are higher in HIV + patients who had adverse reactions to SMX as compared with that of HIV+ patients who did not have such a reactions ($P<0.01$). This difference is more apparent with respect to IgM ($P<0.01$). Other controls did not show any detectable anti-SMX IgM, or IgG.


2.0
1.5
1.0
0.5
0.0
Optical Density
IgG
IgM
IgG
IgM
1
1
2
2

ELISA with SMX-PLL conjugate

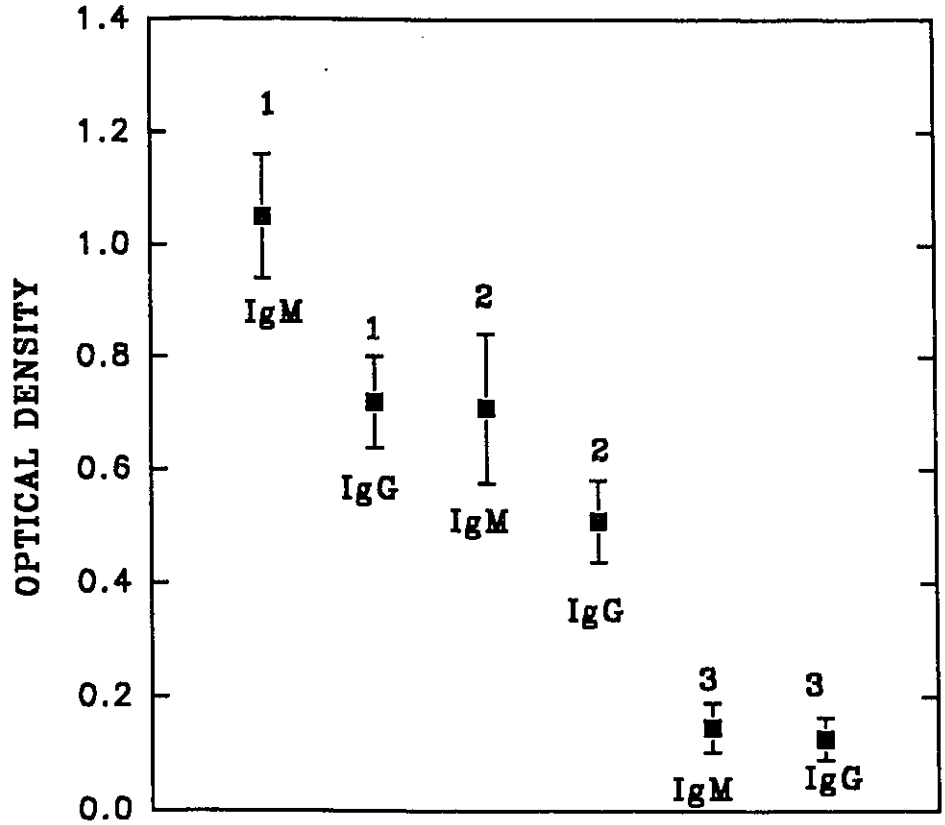
The glutaraldehyde method of conjugation was effective enough for screening for SMX antibodies. However, this method was not easy to be employed for different carriers and the yield of the product was not easily assessed. Therefore, in our second approach poly-L-Lysine, which is not known to react with human serum, was chosen as a carrier. One of the advantages of PLL is that one can chose the desired molecular weight of PLL (in order to mimic what happens in vivo, here PLL with a molecular weight close to human serum albumin was used). PLL was N-acryloylated with acryloyl chloride so that SMX could later be covalently linked to that, this was done in Dr. Roy's laboratory, in the Department of Chemistry, University of Ottawa.

The results of the immunoassay using the Poly-L-Lysine-SMX conjugate for IgG, IgM, and IgE are shown in Table 3. The distribution of ODs are shown in Figure 3. Overall, 92% of HIV-seropositive patients without known adverse reaction to TMP-SMX had either IgG or IgM reacting with SMX. However, the ODs of HIV-seropositive patients with adverse reactions to TMP-SMX were significantly higher than in HIV-seropositive patients without such reactions ($P<0.001$). Anti-SMX antibodies of the IgM class were higher than that of IgG. These results confirm those of ELISA done by the SMX-glutaraldehyde-BSA conjugate. The backgrounds of ODs in the ELISAs with SMX-PLL for IgM and IgG, were approximately 0.20

Table 3. ELISA for the detection of anti-SMX IgG, IgM, and IgE, using SMX-PLL conjugate. Frequency and mean OD values, representing anti-SMX antibodies in normal adults, infants, and HIV-seropositive patients are shown. The ELISA plates were coated with the poly-L-lysine-SMX conjugate to detect anti-SMX IgM, and IgG. Diluted sera of 10 HIV-seronegative adult volunteers, 20 HIV-seropositive patients with a history of adverse reactions to TMP-SMX, and 20 HIV-seropositive patients with no such history, and of 20 infants were used in these evaluation. *P<0.001 compared to healthy adults and infants.

subjects	NO.	IgM	OD ±SD	IgG	OD ±SD	IgE	OD	% of Total
HLT ADT	10	0	0.17 ±.08	0	0.11 ±0.02	0	0.00	0
HIV+,+	20	20*	1.05 ±.19	19*	0.72 ±0.14	0	0.00	97
HIV+,-	20	9	0.71 ±.23	17	0.52 ±0.14	0	0.00	92
Infants	20	0	0.09 ±.01	0	0.09 ±.01	0	0.00	0

Figure 3. Distribution of ODs of anti-SMX IgM, and IgG patients and controls. Distribution of ODs for IgM, and IgG in patients and controls are shown. Anti-SMX IgM, and IgG are higher in HIV-seropositive patients who had adverse reactions to SMX as opposed to that of HIV-seropositive patients with no such reactions. This difference is more significant (P=<0.002) for the IgM results. Other controls did not show any detectable anti-SMX IgM, or IgG.


OPTICAL DENSITY
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
IgM
1
IgG
2
IgM
2
IgG
3
IgM
3
IgG

and 0.10 lower than those observed in SMX-glutaraldehyde-BSA assay. This improvement of the results might be due to replacing PLL instead of BSA and changing the method of conjugation.

IgG subclasses

Different subclasses of IgG vary with respect to their biological activity. These subclasses also may be involved in the pathophysiology of immune reactions, such as, adverse reactions to SMX.

Anti-SMX antibodies of IgG subclasses in patients and controls are shown in Table 4. Anti-SMX IgG antibodies were of IgG_1, and IgG_3 subclasses. The distribution of ODs of IgG subclasses is shown in Figure 4. These subclasses are involve in the activation of complement. IgG_3, for example, is the most efficient subclass of IgG in terms of complement activation.

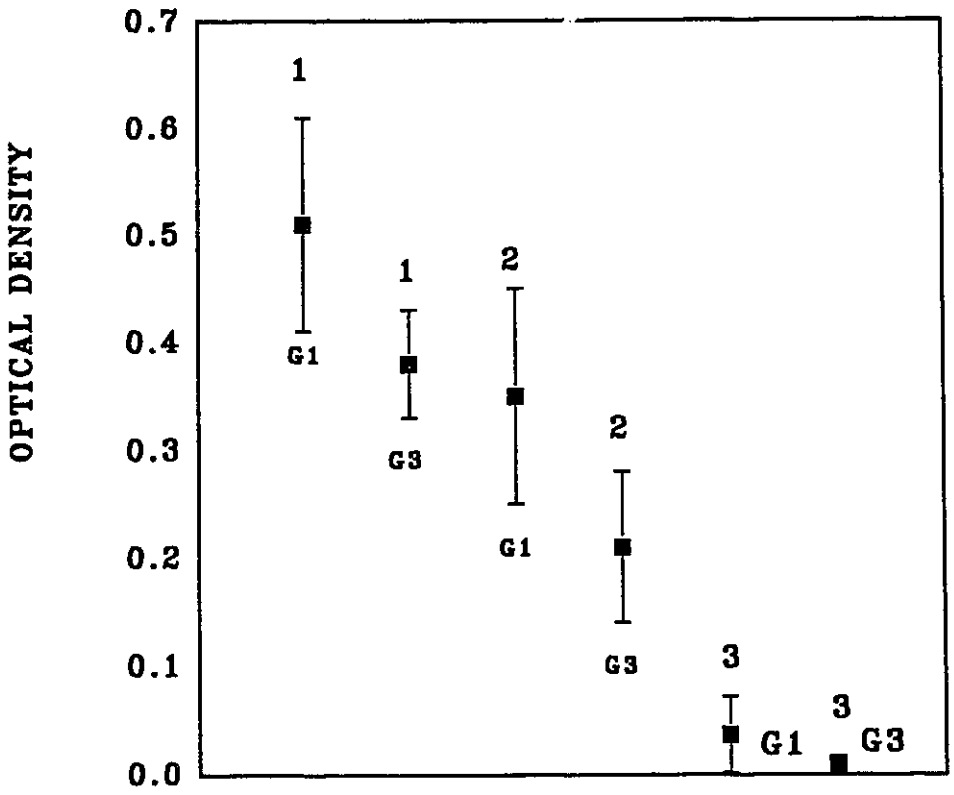
Hapten Inhibition Assay

To determine the specificity of an ELISA, one would need an inhibition assay. In an specific ELISA preincubation of sera with the antigen (in this case hapten) should inhibit further interactions of antibodies with the antigen in the solid phase. Thus, if it is specific, our ELISA should only detect SMX and very closely structurally related compounds. Such an assay may also facilitate the recognition of antigenic determinants of antigens or haptens.

Table 4. ELISA for anti-SMX IgG subclasses, using SMX-PLL. Frequency and mean OD values representing anti-SMX IgG subclasses in infants, normal adults, and HIV-seropositive patients with and without adverse reactions to TMP-SMX. ELISA plates were coated with poly-L-lysine-SMX conjugate. Diluted sera of 10 HIV-seronegative adult volunteers, 20 HIV-seropositive patients with a history of adverse reaction to TMP-SMX, and 20 HIV-seropositive patients with no such history, and 20 healthy infants were used to measure anti-SMX IgG subclasses. *$P<0.001$, compared to healthy adults and infants.

subjects	No	G_1	OD ±SD	G_2	OD ±SD	G_3	OD± SD	G_4	OD ±SD
HLT-ADT	10	0	0.10 ± 0.03	0	.10 ± .02	0	.00	0	.00
HIV+,+	20	19	0.51 ± 0.10	0	.10 ± .01	19*	.38 ± .05	0	.00
HIV+,-	20	17	0.35 ± 0.10	0	.10 ± .02	17	.21 ± .07	0	.00
infants	20	0	.00	0	.02 ± .02	0	.00	0	.00

Figure 4. Distribution of ODs of IgG subclasses (IgG_1 and IgG_3) reacting with SMX, in patients and controls. ODs of anti-SMX IgG_1 and particulary IgG_3 are higher in HIV-seropositive patients who had adverse reactions to SMX (3) as opposed to that of HIV-seropositive patients who did not have such a reactions (2) and that of other controls (1). This difference is more apparent with respect to IgG_3 ($p<0.001$). Other controls did not show any detectable anti-SMX IgG_3 or IgG_3 . 1:Controls(HIV-,-); 2:HIV+,-; 3:HIV+,+ .


OPTICAL DENSITY
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
G1
1
G3
2
G1
2
G3
3
G1
3
G3

The results of the inhibition assay with BSA-glutaraldehyde-SMX for four patient's sera are shown in Table 5 and Figure 5. Patients who showed adverse reactions, were prescribed both TMP and SMX. Therefore, TMP also was used as inhibitor. SMX was able to inhibit the binding of antibodies to the conjugate (BSA-glutaraldehyde-SMX) up to 69%. However, such inhibition was not induced by TMP.

The inhibition assay for poly-L-Lysine-SMX conjugate is shown in Table 6 and Figure 6. This ELISA was performed with sera from three patients who demonstrated high ODs in the ELISA. TMP, SMX, SMX-PLL, sulfisoxazole, sulfanilamide, sulfadiazine, 3-amino-5-methylisoxazole, N'-(2-thiazolyl) sulfanilamide, N'-Acetylsulfanilamide, were used as inhibitors.

SMX, SMX-PLL, sulfadiazine, (2-thiazolyl) sulfanilamide, N'-acetyl- sulfanilamide and sulfisoxazole were able to inhibit antibody binding by up to 80%. Sulfinamide, 3-amine-5-methylisoxazole, PLL, and TMP were not able to inhibit the antibody binding.

In a high concentration (1000 nM), inhibition for SMX (88%) , and the N-acetylsulfanilamide (86%), for exceeded that observed with SNM and 3-amino-5-methylisoxazol were about (23%). SNM and 3-amino-5-methylisoxazol, however, are both present in the SMX structure (appendix II).

The results of inhibition assay suggest that the antibodies detected in our ELISA assays recognize the entire

Table 5. Optical densities in the ELISA after pre-incubating with various compounds. To test for the specificity of the ELISA for the detection of anti-SMX antibodies, the sera of 4 patients with high IgM levels were used in the inhibition assay done by the BSA-SMX-glutaraldehyde method. OD of the ELISA before and after two hour pre-incubation at 37°C with SMX, TMP, and Glycine were determined.

Patients	SMX	TMP	Glycine	Test
No.1	0.32	1.00	1.11	1.23
No.2	0.47	1.25	1.30	1.41
No.3	0.39	1.29	1.35	1.49
No.4	0.53	1.40	1.42	1.65

Figure 5. Inhibition assay for the detection of antibodies to SMX using the BSA-SMX-glutaraldehyde conjugate ELISA protocol, with SMX and TMP as inhibitors. ELISA for the anti-SMX IgM was done before and after incubation of sera with the above mentioned compounds. Sera from 4 patient with high levels of anti-SMX of IgM isotype were selected for this evaluation. Decrease in measured antibody levels exceeded 60% following incubation with SMX, as compared with 20% following incubation with TMP.

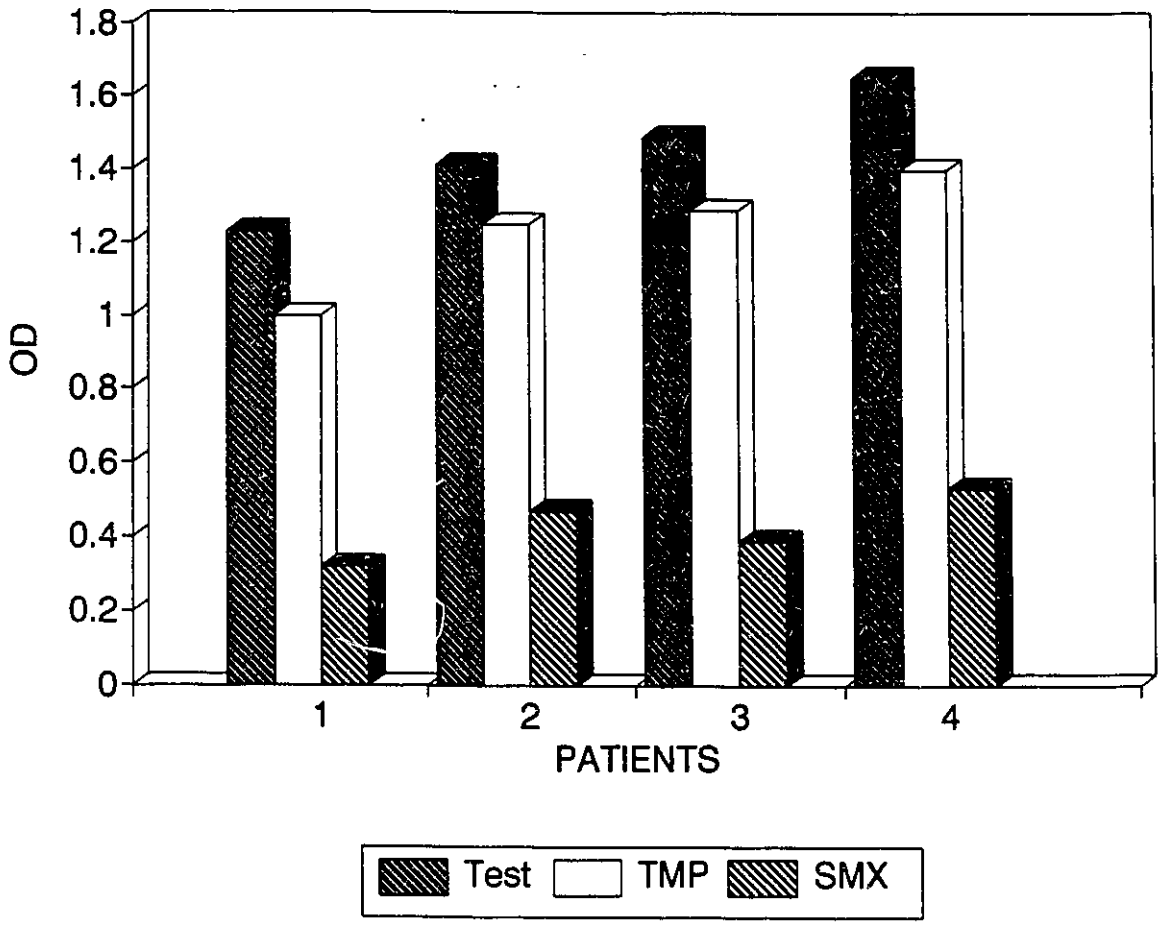
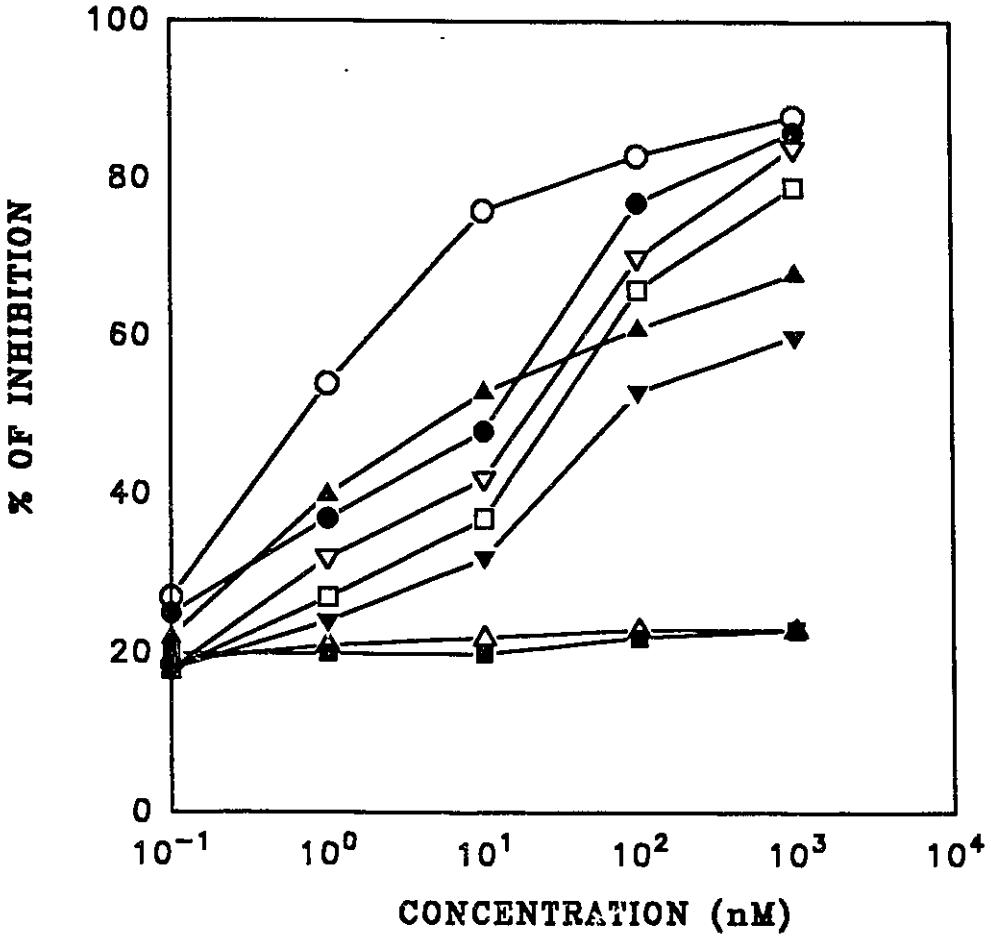

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD
1
2
3
4
PATIENTS
Test
TMP
SMX

Table 6. Optical Density and per cent of Inhibition with different components using SMX-PLL conjugate. Per cent of inhibitions for different compounds in different concentrations are shown. ELISAs before and after incubating positive sera with different compounds were performed, inhibition (%) was calculated by comparison to the control experiment.

quantity of component	0.00 nM	0.10 nM	1.00 nM	10.00 nM	100.00 nM	1000.0 nM
SMX (OD)	1.05	0.78	0.51	0.29	0.22	0.17
% INH,SMX	0%	27%	54%	76%	83%	88%
NACSN (OD)	1.05	0.80	0.68	0.57	0.28	0.19
%INH,NACSN	0%	25%	37%	48%	77%	86%
SDZ (OD)	1.05	0.87	0.75	0.63	0.35	0.21
%INH, SDZ	0%	18%	32%	42%	70%	84%
NTS (OD)	1.05	0.87	0.81	0.73	0.52	0.45
%INH,NTS	0%	18%	24%	32%	53%	60%
SMX-PLL (OD)	1.05	0.87	0.78	0.68	0.39	0.26
%INH,SMX-PLL	0%	18%	27%	37%	66%	79%
SNM (OD)	1.05	0.85	0.85	0.85	0.83	0.82
%INH,SNM	0%	20%	20%	20%	22%	23%
3A5MI(OD)	1.05	0.86	0.84	0.83	0.82	0.82
%INH,3A5MI	0%	19%	21%	22%	23%	23%
SFZ (OD)	1.05	0.83	0.65	52%	0.44	0.37
%INH,SFZ	0%	22%	40%	53%	61%	68%

Figure 6. Per cent of inhibition, for different compounds SMX (○), NSCSN (●), SDZ (▽), NTS (▼), SMX-PLL (□), SNM(■), 3A5MI (△), and SFZ (▲), used in inhibition assay, using SMX-PLL conjugate. ELISAs before and after incubation with inhibitors were performed to calculate per cent of inhibition.


100
80
60
40
20
0
% OF INHIBITION
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
CONCENTRATION (nM)

molecule of SMX and since SMX alone was able to inhibit the antibody interaction, antibodies reacting with SMX were not likely to have a major interaction with the carrier protein used to design the ELISA plates.

RESULTS OF THE CELLULAR RESPONSE TO SMX

Upon exposure to an antigen, lymphocytes may undergo proliferation which could be assayed by ^{3}H-thymidine incorporation and by the CellTiter assay.

^{3}H-Thymidine Incorporation and CellTiter Assay

^{3}H-Thymidine incorporation and CellTiter assay were done for 12 HIV-seropositive patients six of which had a history of adverse reaction to TMP-SMX, one HIV negative patient allergic to SMX, and six healthy adults.

Peripheral mononuclear cells (500,000) were incubated with different compounds at different concentrations.

The results of the two methods were concordant. Cells of HIV-seropositive patients and healthy adults incubated with PLL, sulfanilamide, TMP, RPMI-FCS, Galactose-PLL, and SMX-PLL, did not show any proliferation. Cells of the allergic patient, when incubated with SMX-PLL in concentrations higher than 10 μg per 200 μl of media containing 500000 cells, showed a proliferation higher than that of PWM.

The results of lymphocyte incorporation of thymidine are

shown in Table 7, and Figure 7 and the results of Celltitre assay are shown in Table 8. Cells of patients did not proliferate when cultured with PLL. This supports the specificity of this assay and may also support our choice of PLL as a suitable carrier in such experiments. Cells of HIV-seropositive patients with adverse reactions to SMX, did not show any proliferation upon incubation with SMX-PLL as well as other compounds used in the assay. Although less well than cells isolated from healthy adults, they did proliferate when incubated with PWM. This, together with the fact that these patients had a remarkable amount of anti-SMX antibodies, may suggest that SMX had induced a predominantly humoral responses in these patients.

Lymphocytes of one HIV seronegative patient allergic to SMX showed proliferation when incubated with SMX-PLL in both ^{3}H-thymidine incorporation and in CellTiter assay. The dose-response curve for antigen stimulation of this patient is shown in Figure 8. The results of anti-SMX antibodies and total Igs in the supernatants are shown in Tables 9 and 10, and the results of controls used for standard curve generation for the measurement of total Igs are shown in Table 11. Anti-SMX antibodies in the supernatants are shown in Figure 9 and that of total Ig in Figure 10 (since they were not higher than negative controls, and results are very similar, positive and negative sera were used as positive and negative controls). None of the supernatants show an OD for the anti-SMX antibody

Table 7. The results of lymphocyte proliferation assay (Lymphocyte incorporation of thymidine). Lymphocyte were incubated with 10.00 μg of SMX, TMP, SNM, and SMX-PLL, for 7 days and upon pulsed-labelling with tritiated ^{3}thymidine for 16 hours, cpm was determined by scintillation spectrometry. Results are plotted as the mean of triplicates. Lymphocytes from normal adults, HIV-seropositive patients with a history of adverse reaction to SMX and HIV-seropositive patients with no such reactions did not show a significant proliferation, whereas lymphocytes of an HIV-seronegative individual with a known allergic reaction to SMX demonstrated a high level of lymphocyte proliferation when incubated with 10 μg of SMX-PLL.

Subjects	RPMI	PWM	PPL	SMX	SMX-P	SNM	TMP
HIV+,-	4300	11100	4970	4130	4980	4520	4640
HIV+,+	2600	12100	2300	1800	2300	1600	1700
Healthy controls	6900	14180	2500	4900	6700	5400	6150
Allergic	7812	14500	8600	5120	20080	7322	5238

Figure 7. ^{3}H-Thymidine incorporation of 500000 mononuclear cells after incubation with 10.00 μg/well of SMX-PLL, SMX, SNM, TMP, and PLL. PWM (1:100 in media) and RPMI were used as positive and negative controls. Results of an HIV-seronegative allergic patient are compared with that of HIV-seropositive patients with adverse reaction to SMX and controls.

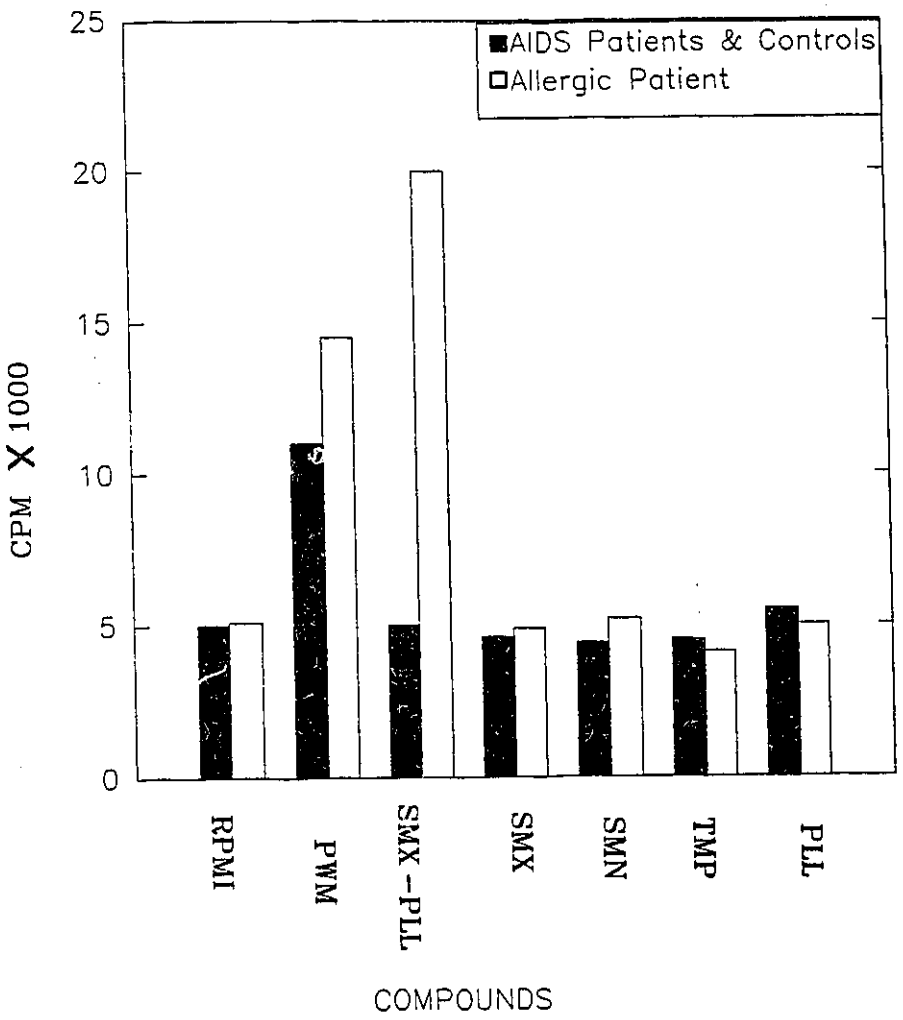

25
20
15
10
5
0
CPM X 1000
AIDS Patients & Controls
Allergic Patient
RPMI
PWM
SMX-PLL
SMX
SMN
TMP
PLL
COMPOUNDS

Table 8- CellTiter assay of antigen stimulation of 500,000 lymphocyte of tests and controls. CellTitre Proliferation assay of patients and controls' lymphocytes. Lymphocytes of 6 HIV-seropositive patients with a history of adverse reaction to SMX, six HIV-seropositive patients without such reactions, six normal adults, and one HIV-seronegative individual with a known allergic reaction to SMX were evaluated. Results are shown when the concentration of compounds was 10 μg/well.

subjects	HIV+,+	HIV+,-	HLT-ADT	ALG
Number	6	6	6	1
RPMI	0.014 ±0.004	0.013 ±0.005	0.018 ±0.003	0.015
PWM	0.190 ±0.003	0.187 ±0.009	0.220 ±0.010	0.239
SMX	0.016 ±0.005	0.017 ±0.003	0.017 ±0.004	0.020
SMX-PLL	0.017 ±0.002	0.023 ±0.002	0.020 ±0.003	0.171
TMP	0.020 ±0.003	0.022 ±0.004	0.018 ±0.005	0.016
SNM	0.016 ±0.004	0.015 ±0.003	0.016 ±0.005	0.019
G-PLL	0.019 ±0.003	0.022 ±0.003	0.016 ±0.005	0.020

Figure 8. Dose-respond curve for antigen stimulation of 500,000 lymphocyte of a patient with a known allergic reaction to SMX. Cpm was determined by scintillation spectrometry. Results are plotted as mean of triplicates. Cpm for SMX-PLL was higher than that of SMX, TMP, SNM, and PLL.

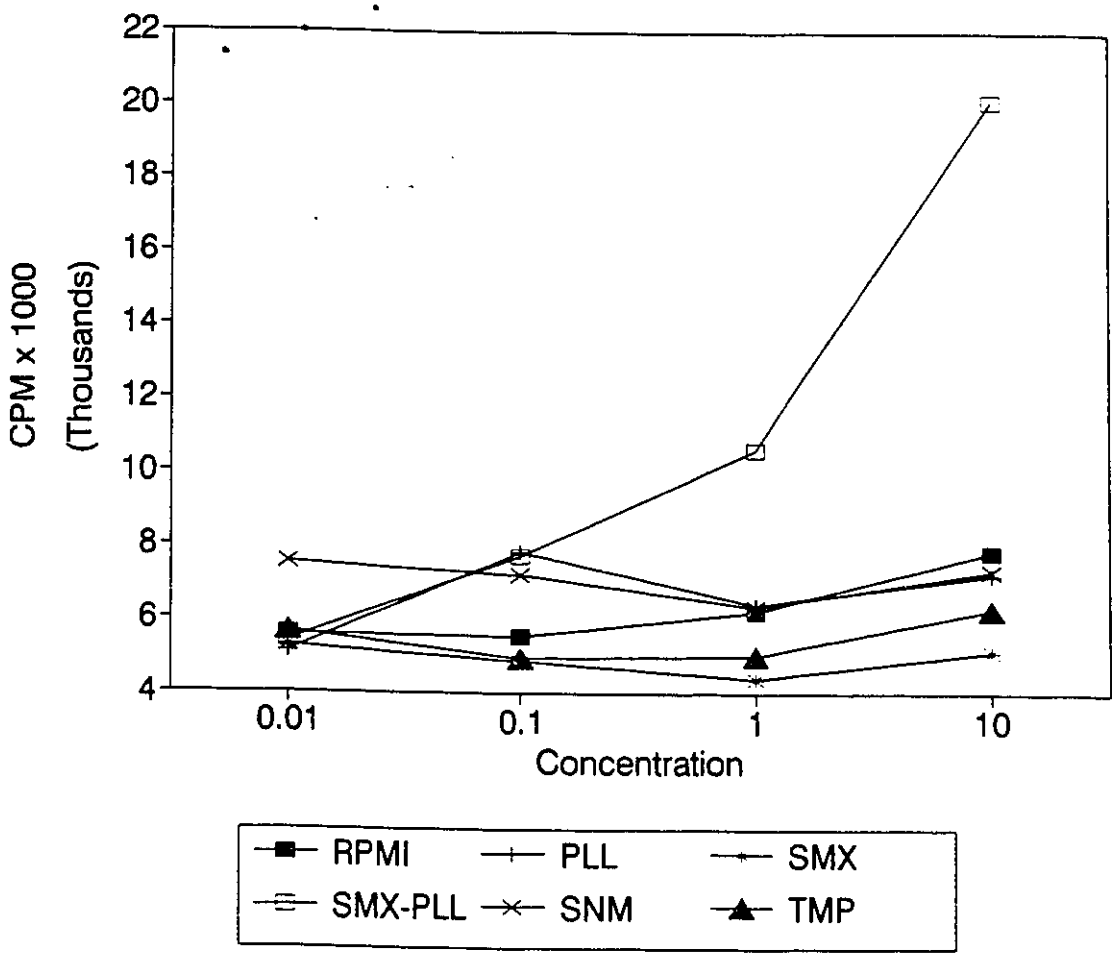

22
20
18
16
14
12
10
8
6
4
CPM x 1000
(Thousands)
0.01
0.1
1
10
Concentration
RPMI
PLL
SMX
SMX-PLL
SNM
TMP

Table 9. Anti-SMX antibodies in the supernatant of a lymphocyte proliferation assay. Supernatant of the lymphocyte proliferations after different stimuli were assayed for anti-SMX antibodies. None of the supernatants showed an OD for the anti-SMX antibody higher than that of RPMI, except the cells stimulated with PWM. This, however, might have been due to non-specific binding of antibodies to plates.

	N	RPMI	PWM	SMX	SMX-PLL	SNM	TMP	PLL-G
HIV+,+	6	.050 ± .004	.090 ± .003	.028 ± .004	.031 ± .002	.020 ± .004	.020 ± .003	.020 ± .005
HIV+,-	6	.040 ± .005	.087 ± .005	.032 ± .006	.029 ± .005	.025 ± .003	.024 ± .004	.022 ± .004
HLT ADT	6	.051 ± .003	.095 ± .005	.030 ± .004	.027 ± .002	.024 ± .005	.031 ± .003	.026 ± .004
ALG	1	.049	.098	.030	.028	.029	.035	.034

Table 10. Total Igs in the supernatant of the cells of the tests and controls incubated with different drugs. Plates coated with anti-IgM and anti-IgG were used for the ELISA, HRP conjugated anti-IgM and anti-IgG were later used in this assay. *, c.c.: Conjugate Control

	SMX	SMX-PLL	TMP	SNM	PWM	RPMI	PLL	c.c.*
HLT ADT	0.10	0.09	0.07	0.09	0.10	0.06	0.07	0.10
HIV+,-	0.08	0.09	0.08	0.09	0.09	0.09	0.10	0.07
HIV+,+	0.09	0.07	0.08	0.10	0.06	0.06	0.07	0.06
ALG	0.09	0.08	0.06	0.07	0.10	0.07	0.08	0.08

Table 11. ELISA with a normal serum used as standard. The results of ODs of serial dilutions of serum of a healthy adult as standard curve used for the measurement of total Igs in the supernatant. Coated plates with Igs were used in the ELISA.

serial dilution	1/10	1/20	1/40	1/80	1/160	1/320	1/360
OD	0.45	0.29	0.22	0.16	0.10	0.06	0.01

Figure 9. The mean of anti-SMX antibodies in the supernatants of the wells of the lymphocyte proliferation assay in tests and controls. None of the results were higher than that of media controls. The sera of a patient with a history of adverse reaction to SMX and a healthy adult were used as positive and negative controls.

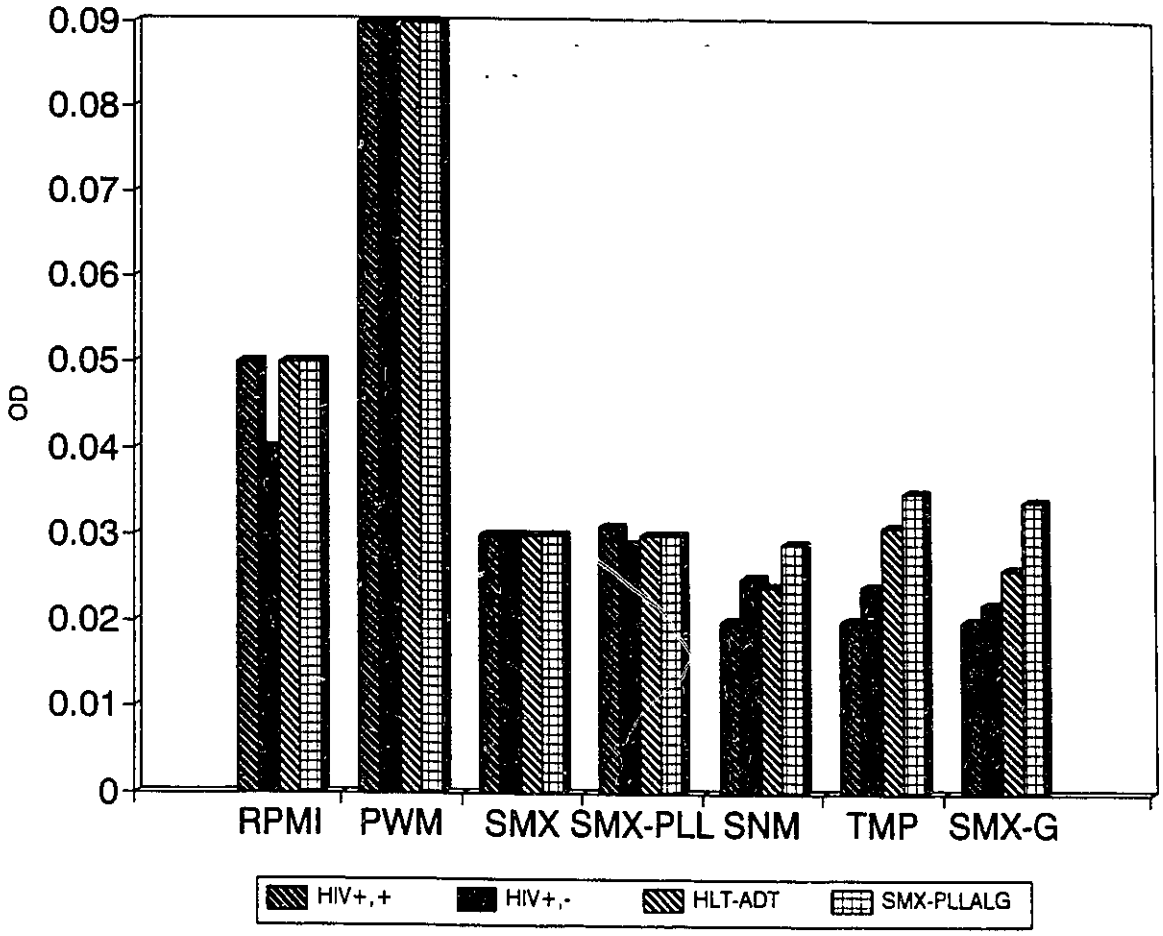
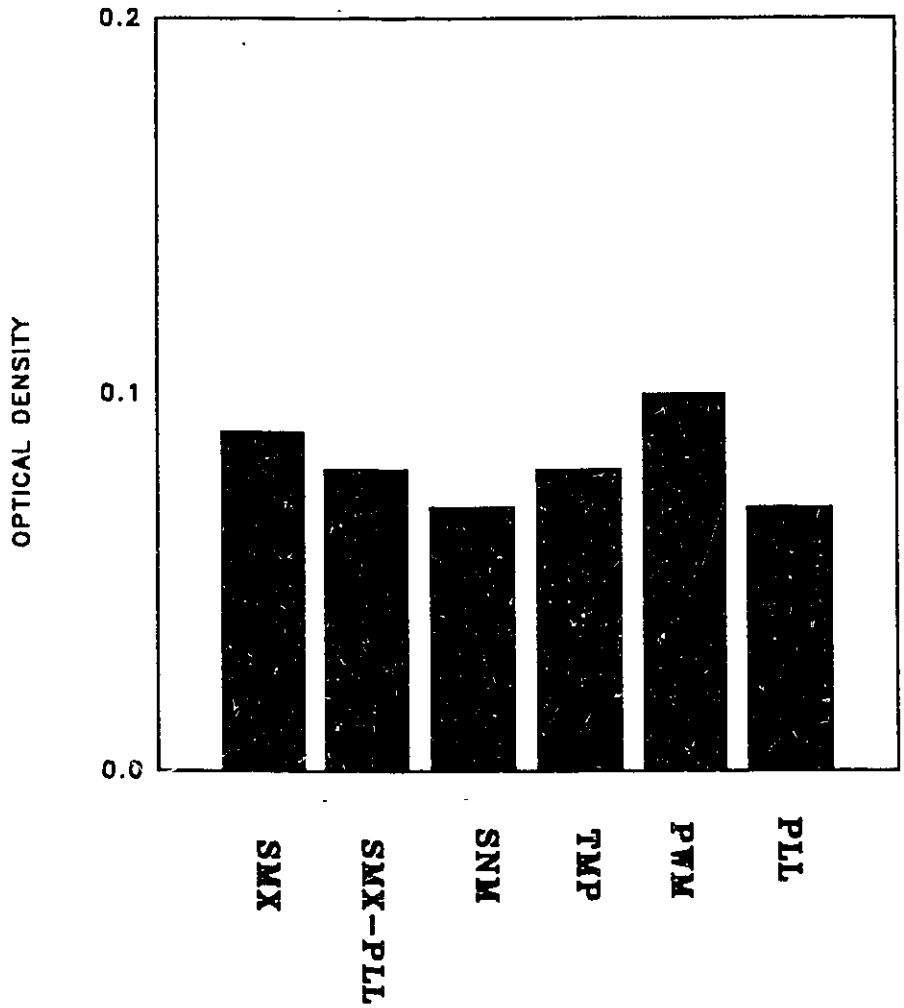

0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
OD
RPMI
PWM
SMX
SMX-PLL
SNM
TMP
SMX-G
HIV+,+
HIV+,-
HLT-ADT
SMX-PLLALG

Figure 10. Total Ig. in the supernatant of the wells of proliferation assay (^{3}thymidine incorporation). The serum of a normal adult was used as standard. Plates were coated with antisera to human Ig. , incubated with the serum of healthy individual, and antisera to human Ig. (conjugated with HRP) were added.


OPTICAL DENSITY
0.2
0.1
0.0
SMX
SMX-PLL
SNM
TMP
PWM
PLL

higher than that of RPMI except the cells stimulated with PWM. This, however, might be due to non-specific binding of antibodies to plates. Total Igs also were not higher than conjugate controls (negative controls) except for the supernatant of cells stimulated with PWM. Generally speaking, the measurement of Ig in cell culture supernatant, is considered a sensitive method. In spite of having appropriate negative and positive control, it is possible that the assay was not sensitive enough to detect the Ig in the supernatant.

This might be due to few B cell clonotypes pertaining to SMX in peripheral blood of our patients, or because of lack or decrease of some cytokines in an in vitro assay for HIV-seropositive patients.

DISCUSSION

Adverse reactions to cotrimoxazole include rash, fever, gastrointestinal symptoms or haematological abnormalities, which may hamper treatment of pneumocystosis or other infections in patients with AIDS. The majority of adverse reactions to cotrimoxazole are secondary to SMX and not to TMP (Levy,1989; Van Der Ver,1991) . It is not yet clear whether the increased frequency of side effects are due to drug toxicity or an abnormal immunological reaction to SMX. The prevalence of reactions in AIDS patients is related to the dose of cotrimoxazole (Levy,1989; Van Der Ver,1991). Patients with Pneumocystosis are treated with high dose cotrimoxazole. However, immunosuppressed HIV-seronegative patients on high dose cotrimoxazole have a lower frequency of adverse reactions (15%) (Hughes et al, 1978; and Winston et al, 1980).

Recently Van De Ven et al (1991) suggested that HIV-seropositive individuals can have alterations in the metabolism of sulfonamides, and that excess hydroxylamine derivatives may induce toxicity to sulfa compounds.
Antibodies against SMX have been described in patients with adverse reactions to this drug, both in HIV-seropositive and seronegative populations (Ressler,1987; Levy,1989). This study shows that antibodies that recognize SMX conjugates are present in the majority of HIV-seropositive patients, whether they have experienced adverse effects to SMX or not, but higher antibody levels are observed in patients who did not tolerate therapy than in those who did. It is known that 40%

of HIV-seropositive individuals tolerate SMX without adverse reactions (Levy,1989).

In our study, many individuals with detectable anti-SMX antibodies had undergone treatment with this drug without side effects. Thus, it would appear that a low-level of antibodies to SMX can be tolerated without overt symptoms, and that there may be a critical level of antibodies to SMX necessary to develop a reaction (rash and fever). The humoral immune response to SMX is characterized by increased levels of specific IgM, IgG_1, and more apparent with respect to IgG_3. The detection of anti-SMX IgM antibodies identified patients with drug reactions better than the IgG antibodies. The presence of IgM antibodies to SMX, could also predict the development of adverse drug reactions better than IgG antibodies. Production of certain cytokines is required in order for the IgM response to be directed to a subsequence IgG subclass response (class switching). These cytokines are produced upon recognition of the antigenic system by T cells. T cells, however, will only respond to immunogens processed by antigen presenting cells. The presence of anti-SMX specific IgG_1 and IgG_3 subclasses in patients' sera suggests immunoglobulin class switching,has occured,likely with the development of antibody affinity maturation and the involvement of T-cell help. Furthermore, increased production of interleukin-10 (Benjamin et al, 1992), which has been suggested in AIDS patients, may contribute to the induction of

an exaggerated humoral response to SMX in AIDS patients. Involvement of T cells also suggests that SMX is somehow processed by the antigen presenting cells.

IgG_3 represents only 6% of total IgG in the serum, but it made up more than 30% of the antibodies against SMX in this study. This IgG is the most efficient subclass to initiate complement activation (Papadea et. al.,1990). The presence of high levels of this IgG subclass specific to SMX suggests that it may have a role in adverse reactions to SMX among AIDS patients, and that complement could be involved in these reactions. IgG_1, and IgG_3 bind to neutrophils and monocytes via Fc receptors (Tizard, 1988; Roitt, 1988), so it is possible that SMX bound to cellular antibodies could be cytotoxic and could result in the release of mediators of inflammation.

HIV-seropositive patients are predisposed to diseases caused by immunologic injury, and have polyclonal B cell activation, which may be due to several factors, including decreased T suppressor cells and, EBV, HHV-6, CMV, and other opportunistic infections. The increased levels of polyclonal IgG, IgA, perhaps increased IL-10 secretion and immunecomplexes in these patients may contribute to their susceptibility to type II and type III hypersensitivity reactions.

The results of the lymphocyte proliferation assays in HIV-seropositive patients suggest that adverse reactions to

SMX in HIV-seropositive patients are mainly humoral in nature. The cell-mediated immunity plays a role in the adverse reactions to SMX by providing help for the Ig class switching. However, in an in vitro reaction lymphocytes of HIV-seropositive patients may need some supplement to respond properly. Thus, failure of in vitro production of detectable amount of anti-SMX antibodies by HIV positive patients may be due to the insufficient levels of some growth factors such as IL-2. In the serum of our HIV seronegative patient with known adverse reaction to SMX, no detectable anti-SMX antibody was present while the lymphocyte proliferation assay for SMX-PLL of the same patient was positive . This could suggest the involvement of CMI reactions to SMX in this patient.

In the design of conjugates , we selected a carrier molecule, which is not usually recognized by the immune system. Poly-L-Lysine (PLL) is a weak immunogen, does not usually elicit a T-cell response, and has been used previously as an inert carrier (Tatake et al, 1991).

The inhibition experiments confirmed the antibodies that recognize the SMX conjugates were specific for SMX. In addition, they suggest that the immunogenic determinant of SMX is not present in either sulfanilamide or 3-amino-5-methylisoxazole, and that the antibodies to SMX recognize both the sulfanilamide and 3-amino-5-methylisoxazole moieties. Further work is underway to better characterize these determinants.

The mechanism of the development of side effects to SMX is complex, involving both toxic and immunologic components. It may be that severe reactions are an effect of high drug levels interacting with protein carriers, which may lead to a symptomatic humoral immune response only when antibodies have exceeded a certain threshold, which may vary from one individual to another.

These results suggest that sulfa compounds not reacting with anti-SMX antibodies could be alternative drugs for the therapy of opportunistic infections requiring the administration of sulfonamides. The inhibition assay needs to be expanded so that the results may facilitate the development of drugs with less adverse reactions.

FUTURE WORK

My work provides improved techniques such as ELISA, hapten inhibition assay , and proliferation assay for the study of adverse reactions to SMX.

The ELISA described in this thesis, may be used in patients to identify individuals at risk and perhaps to predict adverse reactions to SMX, particularly in AIDS patients.

The hapten inhibition assay can be employed to map antigenic determinants of SMX. It may then be used to design sulfa compounds with less adverse reactions in patients with hypersensitivity to SMX.

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APPENDIX

APPENDIX I

CLASSIFICATION OF ADVERSE REACTIONS TO DRUGS

Overdosage. The amount of the drug being used may be crucial and in different dosage the drug may have different effects. For instance, sulfamethoxazole is used to treat urinary tract infections, but in high doses may accumulate in urine, forming crystals and may induce renal failure (De Weck and Bundgaard, 1983 et. al.,1983)

Intolerance. Intolerance is an untoward reaction because of increased drug effects not related to overdosage (e.g.low doses of quinine may cause cinchonism) (De Weck and Bundgaard, 1983; Anderson and Adkinson, 1987).

Idiosyncrasy. Idiosyncrasy is an increased drug effect which is not related to drug's mechanism of action (e.g. haemolytic anemia caused by primaquine in 6 GPD deficient patients). The term idiosyncrasy is not used in immunological reaction to drugs (De Weck and Bundgaard, 1983; Anderson and Adkinson, 1987).

Side effects. Undesired but unpreventable effects of a drug are called side effects (e.g. sedative effects of most antihistamine drugs) (De Weck and Bundgaard, 1983; Anderson and Adkinson, 1987).

Secondary effects. Secondary effects refer to effects of a drug due to usual mechanisms but on targets other than the one that the drug is being used for (e.g. effects of antibiotics on bacteria of the gut) (De Weck and Bundgaard,

1983 et. al.,1983,1983; Anderson and Adkinson, 1987).

Drugs interactions. Concomitant treatment with some drugs can cause adverse reactions due to interaction of these drugs or because of other unknown mechanisms. A drug may enhance the effectiveness or toxicity of another drug. TMP-SMX and Methotrexate, for example, are shown to have hazardous interactions (De Weck and Bundgaard,1983; Anderson and Adkinson, 1987; Cribb et. al.,1990,1991).

Allergic reactions to drugs. Allergic reactions to drugs are reactions in which cellular and/or humoral immunity are involved (De Weck and Bundgaard, 1983; Anderson and Adkinson, 1987). This point was discussed in detail in this thesis.

Pseudoallergic reactions to drugs. Nonspecific release of mediators can result in a reaction that mimics the clinical symptoms of anaphylaxis (Tizard,1988). These reactions are not IgE mediated. They are in some cases the result of the effect of drugs on immune tissues, for example, anaphylactoid or anaphylaxislike reactions to the radiocontrast media or iodinated contrast media, dyes such as fluorescein and bromosulphalein, and aspirin (De Weck and Bundgaard, 1983 ; Anderson and Adkinson, 1987; Tizard, 1988). Some drugs may initiate mast cells degranulation by non-immunological mechanisms, such as polymyxin B, heroin, codeine, and morphine (De Weck and Bundgaard, 1983 ; Anderson and Adkinson, 1987; Tizard, 1988).

Pseudoallergic reactions are sometimes referred as

idiosyncratic reactions. Aspirin and associated non-steroidal anti-inflammatory agents are able to produce pseudoallergic reactions by means of interference with arachidonic acid (De Weck and Bundgaard, 1983).

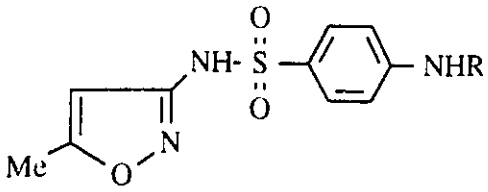
Some of these reactions are due to direct activation of complement (De Weck and Bundgaard, 1983; O Neil,1991).

APPENDIX II

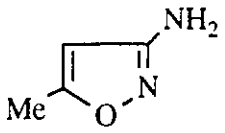
CHEMICAL STRUCTURES OF SMX-PLL AND COMPOUNDS USED IN INHIBITION ASSAY:

Chemical structures of SMX-PLL as well as main compounds used in inhibition assay and also in T cell assays are shown in figure 11.

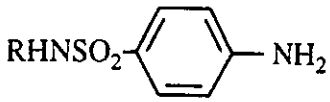
Figure 11. Chemical structures of SMX-PLL as well as main compounds used in inhibition assays and in T cell assays.



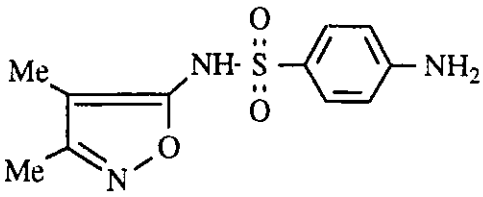
R = H, Sulfamethoxazole (SMX)
R = $COCH{=}CH_2$, N-Acryloylated SMX



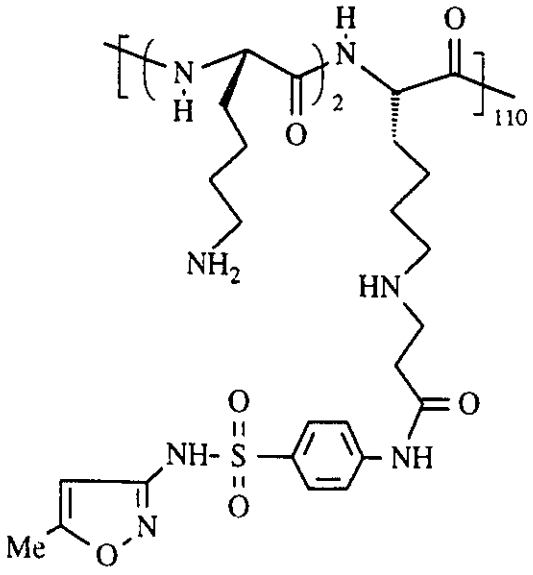
3-Amino-5-methylisoxazole (3A5MI)



R = H, Sulfanilamide (SNM)
R = $COCH_3$, N^1-Acetyl SNM (NACSN)



Sulfisoxazole (SFZ)



Poly-L-Lysine-SMX