Biologicals : principles, techniques and mechanisms of action

B. E. Sands

Gastrointestinal Unit and Center for the Study of IBD, Massachusetts General Hospital, Boston, U.S.A.

Abstract

Biological agents for the treatment of IBD are the result of both the explosion of knowledge precipitated by the techniques of molecular biology, and by the ability to use these same techniques to produce agents. Thus, there has been a greatly facilitated translation of basic knowledge into clinical therapy. An astounding number of biologic agents are currently in development for the treatment of IBD and other immune-mediated conditions. These include native microbiologic preparations isolated for beneficial properties, recombinant cytokines and anticytokines, monoclonal antibodies, antisense oligonucleotides, and in the future, somatic gene therapy. This work seeks to describe the principles and techniques of biologic agent development, as well as prime sites of action targeted by these agents.

Recent advances in the techniques of molecular biology have made possible unprecedented progress in the treatment of many conditions. The techniques of molecular biology have provided new methods of drug discovery and at the same time have elucidated new therapeutic targets. Most notable has been the progress made in the treatment of chronic inflammatory and immune mediated conditions, including inflammatory bowel disease. This paper is intended to highlight the methodological principles behind biologic agents, methods of discovery and production, and to highlight potential therapeutic targets for these new agents. (Acta gastroenterol. belg., **2001**, 64, **165-169**).

Principles of molecular biology

The central dogma of molecular biology is that the physical structure of DNA provides the genetic code to determine composition and structure of every cell in every tissue. Codons, consisting of three sequential base pairs, encode for specific amino acids strung together in peptide chains. The double-stranded DNA, in the process of transcription, serves as the template for messenger RNA. Messenger RNA, in turn, shuttles this message from the nucleus to the ribosome within the cytoplasm. Messenger RNA, finally, provides the encoded message enabling the ribosomal machinery to manufacture the strings of amino acids comprising each protein. Each protein possesses secondary structure by virtue of disulfide bonds between cysteine residues, and tertiary structure, such as alpha chain and beta pleating. These structural aspects of proteins are essential to specific function. Tightly regulated control of transcription is critical for normal cellular functioning. Binding of nuclear factors to nucleotide sequences in the upstream promoters or intronic regions is highly specific, thereby modulating gene expression. Regulated expression of nuclear factors is responsible for the specifities of cellular differentiation, growth and apoptosis that permit normal function and homeostasis in all tissues. Modifications in the tail sequences of messenger RNA may affect longevity of the transcript, with additional consequences for gene expression. Finally, post-translational modifications of peptides in the endoplasmic reticulum and the Golgi apparatus may markedly alter protein function.

Definition of biologic therapies

Biologic therapies have long been a part of the medical armamentarium, dating at least to the use of vaccinia for immunization against smallpox. In the United States biologic therapies come under the regulatory guidance of the Food and Drug Administration as mandated by the Federal Food, Drug and Cosmetic Act (title 21). Traditional biologic therapies have long included therapeutic sera, toxins and antitoxins, as well as live attenuated or killed organisms as immunizations. Jurisdiction for regulation of biologic agents in the FDA falls under the purview of the Center for Biologics Evaluation and Research (CBER). More recently, the family of biologic therapies has grown to include recombinant peptides and proteins, antibody based therapies, and cell and gene therapies. For purposes of considering mechanism of action, nucleic acid based therapies may also be considered in this family, although strictly speaking these are defined chemical compounds.

Compared to the development of most compounds based drugs, development of biologic agents has been relatively rapid. Traditionally, drugs have most often been discovered by large-scale screening of libraries of compounds to identify parent drugs. Parent compounds often require chemical manipulation to produce secondary compounds with improved characteristics. This process is usually long and expensive. In contrast, biologic agents are often developed by selection of candidate peptides of identified physiologic function. Therefore the time for preclinical development may be relatively short and potentially less expensive (Table 1). Peptides that have been used as therapeutics include recombinant cytokines, cytokine receptor antagonists, hormones, and chimeric and humanized antibodies. Possible agents of the future include antisense oligonucleotides and somatic transgenic vaccination, essentially gene therapy (1).

Corresponding author : Sands B. E., Gastrointestinal Unit, Massachusetts General Hospital, 55 Fruit Street, Boston, Massachusetts 02114, U.S.A..

Table 1. — **Comparison of compound-based drugs and biological agents (Adapted from Sands (1) with permission)**

	Compound-based drug	Biologic Agent
Composition	"Small molecule" compound	Peptide, protein, RNA, DNA
Route of administration	Oral, parenteral, topical	Typically parenteral
Discovery and Development	Usually by large-scale screening of compound libraries to identify parent drugs ; chemical manipulation to alter characteristics	Choice of target defined by mechanism of disease. Development often by recombinant techniques
Cost	Expensive to develop, less expensive to produce	Relatively less expensive to develop, more expensive to produce
Time to clinical trial	Relatively long	Relatively brief
Examples	Classic receptor blockers, enzyme inhibitors, corticosteroids, immune modulators	Recombinant cytokines, cytokine receptor antagonists, chimeric and humanized antibodies, antisense oligonucleotides, somatic transgene vaccination

The common feature of all recombinant peptides and proteins is their production in cell based systems. Typically, the gene of interest is cloned into a prokaryotic or eukaryotic expression system. These may include bacteria such as E. coli, yeast or mammalian cells in culture. These cells are utilized as living factories. Depending upon the cell line and the nature of the proteins being produced, cells grown in culture may be harvested and lysed for their proteins or supernatants may be collected for further isolation and purification of large quantities of specific peptides. One potential advantage of using recombinant technology is the ability to produce fully human peptides, identical to those found in man.

Monoclonal antibodies

A particularly useful form of recombinant protein is the monoclonal antibody. This therapeutic modality takes advantage of the binding specificity characteristic of all antibodies. By using recombinant technology, antibodies may be used to pharmacologic effect by virtue of being able to produce large quantities of antibody of a singular binding specificity. Traditional monoclonal antibodies are of murine derivation. Historically, the first monoclonal antibodies were obtained by fusing immortalized mouse myeloma cell lines with splenic cells of mice immunized with the target protein of interest. Isolation of individual cells, called hybridomas, produced immortalized cells that were essentially factories for the production of a single antibody specific to the immunized protein.

The host immune response to murine antibodies was a marked limitation in the utility of monoclonal antibodies as therapeutic agents for repeated use. As monoclonal antibodies were increasingly thought of for their therapeutic potential in chronic diseases, the carryover of murine antigenic determinants has become less desirable. Therefore, through recombinant techniques, the antigen binding specificity of monoclonal antibodies has been preserved by cloning the variable regions of the light and heavy chains onto a backbone of human immunoglobulin. The resulting chimeric antibody is roughly 25 to 33 percent murine sequence, with the remaining sequence human. Nevertheless, antibodies against non-human epitopes may still develop in the course of treatment with chimeric antibodies. Therefore increasing efforts have been directed toward making more fully human antibodies (2). Through computer modeling, the exact determinants of epitope binding specificity may be identified. These so-called complementarity determining regions have been grafted onto a human immunoglobulin backbone through recombinant techniques. The resulting antibody preserves the main points of interaction between antigen and antibody while further decreasing the murine component to roughly five percent. More recently, introducing the human immunoglobulin gene into mice with deleted human immunoglobulin gene has produced fully human antibodies. Such techniques may permit production of less antigenic antibodies. However, it is still conceivable that such antibodies may not be a part of the normal repertoire of healthy human, and may yet generate human anti-human antibodies. Other properties that may alter the pharmacologic effects of monoclonal antibodies include the binding characteristics and the isotype. Antibodies of IgG1 isotype, for example, differ from those of isotype IgG4, with the former capable of fixing complement and effecting antibody-dependent cell-mediated cytotoxicity (ADCC).

Antisense oligonucleotides

Nucleic acid based therapies hold great promise as novel pharmacologic agents (3). Antisense oligonucleotides are in clinical use for indications outside of inflammatory bowel disease, and have been tried for these diseases, as well. Antisense oligonucleotides are single stranded DNA strands designed to bind to a specific RNA message in a complementary fashion. Such DNA-RNA hybrids may directly preclude translation by the ribosomal machinery, or may make messenger RNA susceptible to RNases. In this fashion, precise down-

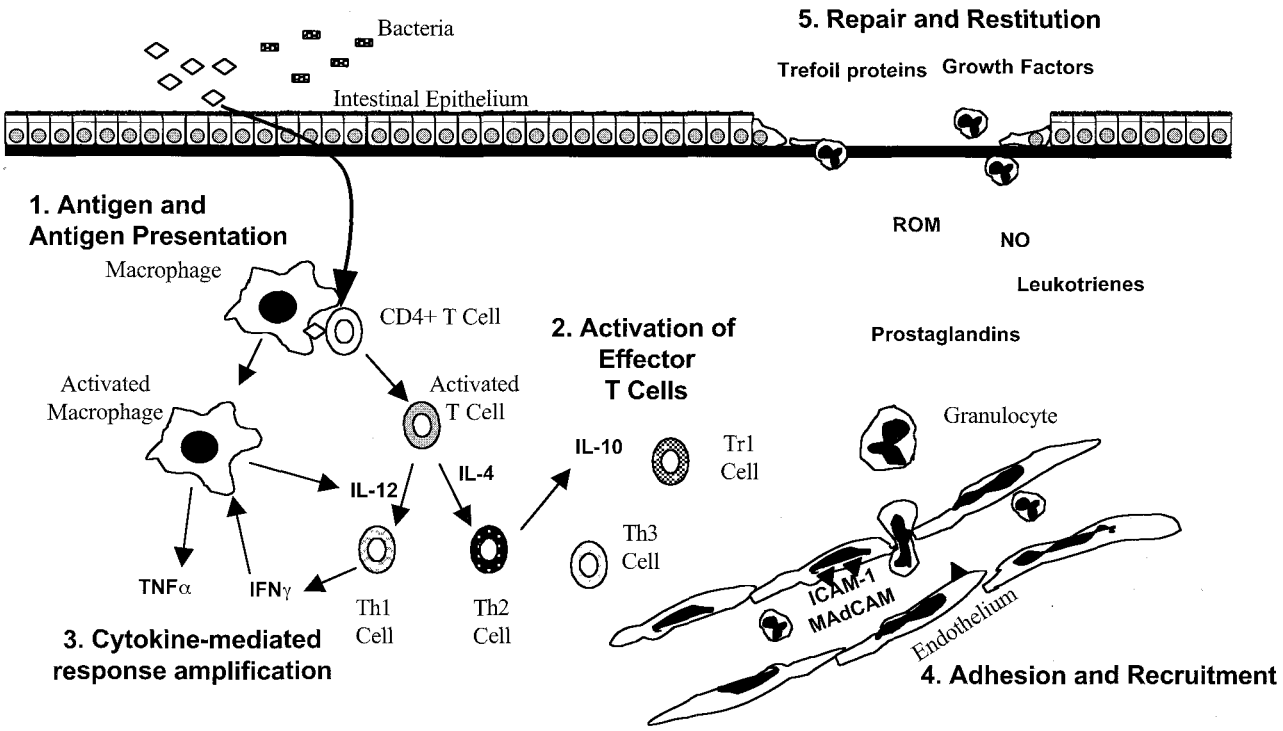

Fig. 1. — Overview of therapeutic targets for biologic agents in inflammatory bowel disease. Adapted from Sands (1) with permission.

regulation of gene expression may be effected. The challenge of utilizing antisense oligonucleotides effectively rests primarily on the ability to deliver sufficient molecules to the site of action. Local delivery systems may prove more effective than systemic delivery, which presently requires parenteral administration.

Mechanisms of action

With increasing knowledge of the pathogenesis of inflammatory bowel disease, many potential therapeutic targets have been identified. Genetic animal models have been particularly fruitful in identifying new therapeutic strategies. Targets for biological agents may be divided into the following areas : 1) antigen and antigen presentation ; 2) activation of effector T cells ; 3) cytokine mediated response amplification ; 4) adhesion and recruitment ; and 5) injury and repair (1) (Fig. 1).

Antigen and antigen presentation

Both Crohn's disease and ulcerative colitis are characterized by a relentless inflammatory response to as of yet unidentified agents or antigenic determinants. Genetic animal models of IBD raised under gnotobiotic conditions have been observed to have absent, attenuated or delayed expression of IBD reversed by the introduction of non-pathogenic intestinal flora. These observations have raised the possibility that in human IBD, the genetically susceptible host may have a pathological inflammatory response to normal flora. This suggests, as well, one means by which antibiotic therapies may be therapeutic in IBD. Should IBD prove to be a pathologic response to a small range of microorganism species, specific therapy could be offered. Until such a discovery occurs, another possible focus might be blocking antigen presentation. Theoretically, specific interventions blocking the expression or binding of the T cell receptor, or of the MHC class II molecule on the antigen presenting cell (4), might prove therapeutic in IBD. Alternatively, peptides with slight modifications of specific antigenic determinants could be used to bind MHC yet prevent recognition by T cells. Such techniques have been used with success in animal models of other immune-mediated diseases.

Probiotic agents may also prove useful in the treatment of IBD (5-7). Probiotic therapy rests on the knowledge that the range of non-pathogenic flora in the intestine is vast, and that species and subspecies vary considerably in their immunologic effects in the intestine. Some flora may upregulate intestinal immune responses, while others effect considerable inhibition of the immune response and maintain normal intestinal tolerance. These effects are dynamic, and may depend upon complex factors within the microflora ecology. Reports of successful treatment with probiotic agents of chronic pouchitis (8), active ulcerative colitis (7) and maintenance of remission in ulcerative colitis (5) have stimulated interest in these extremely safe therapeutics. Additional reports of Saccharomyces boulardii for

maintaining remission in Crohn's disease (9) have provided additional interest. Alternate approaches have been the ingestion of defined carbohydrate supplements capable of fostering the growth of beneficial bacteria in situ (10), in what has been called "prebiotic" therapy.

Activation of effector T cells

CD4+ T cells have long been known to be central to the pathogenesis of IBD. For this reason, modulation of T cell responses has been a prominent goal of new biological therapies. The interaction between antigen presenting cell and the T cell has been likened to an immune synapse. Like nerve synapses, the net effects of receptor binding and post-receptor signaling are complex, and depend upon an integration of not just antigen binding, but a variety of possible second messages, as well. Activation of CD4+ T cells therefore requires not only binding of epitopic determinants in MHC class II to the T cell receptor, but also a second, stimulatory signal (11). Without an appropriate second message, the T cell may fail to be stimulated, but rather may become anergic or die by apoptosis. Classic antigen presenting cells bear one of at least two distinct costimulatory signals, called B7-1 or B7-2, which bind to CD28 on the T cell. Once activated, T cells begin to express CTLA-4 (cytotoxic T cell antigen 4). B7 binds with greater affinity to CTLA-4 than CD28, thereby inhibiting T cell proliferation and activation and dampening the immune response (12, 13). Other costimulatory signals that may be critical include binding of cell bound tumor necrosis factor (TNF) and CD40 to CD40 ligand (14).

Investigational approaches to inhibiting the generation of effector T cells have included the use of soluble MHC class II peptides with antigenic peptide (15). This sort of agent, in the absence of the crucial second signal, could induce tolerance by anergy or deletion of responsive clones, and holds the possibility of a disease modifying effect. Such an approach has not been tried in IBD for lack of identified antigenic determinants, and of a consistent MHC peptide. Approaches aimed toward blocking the second signal seem more promising, and include a fusion protein of CTLA4 and immunoglobulin, called CTLA4Ig, shown to be effective in psoriasis (16). Biologic inhibitors of B7, and of the interaction of CD40-CD40L have also been proposed. Finally, the more blunt approach of using antibodies against CD4 itself have proven to be effective when capable of depleting CD4+ T cells (17,18). These have not entered into clinical use, however, because of concern over persistently low CD4 counts.

Cytokine-mediated response amplification

A confusing abundance of cytokines regulates inflammation in tissues affected by IBD. Only gradually have the significance of these perturbations been sorted out, with many controversies remaining to be untangled. Cytokines modulate the differentiation of populations of T cells, as well as macrophages and other antigen presenting cells. In addition, they serve to amplify the immune response. Key cytokines are likely to include interleukin-2, a T cell growth factor, interleukin-12 and interferon gamma, which shape T helper 1 responses, and interleukin-10 and transforming growth factor beta, which may be essential in the development of downregulatory cells described as having a phenotype of Tr1 (T regulatory cells 1) (19) or Th3 (T helper 3) (20). Pharmacologic manipulation of theses cytokines, by inhibiting the expression or downstream effects of effector cytokines, or by fostering the effects of regulatory cytokines, are effective in animal models of IBD, and may prove effective in man. Proinflammatory cytokines, such as interleukin-1 and tumor necrosis factor, potently amplify the immune response through enormously diverse mechanisms, and are therefore appropriate targets for pharmacologic inhibition in IBD.

Adhesion and recruitment

The lamina propria of normal intestine contains a physiologic amount on inflammation, likely poised to respond quickly to mucosal insult. The marked inflammatory response characteristic of IBD occurs by an influx of macrophages, granulocytes, and lymphocytes, primed and activated. Recruitment of these cells to the mucosa is regulated by a variety of chemokines and adhesion molecules (21). Recruitment occurs through a coordinated series of steps, beginning with a transient tethering of leukocytes to the endothelial surface mediated by selectins present on the leukocyte and the endothelial cell. This induces rolling along the endothelial wall. In the presence of chemokines secreted by activated inflammatory cells already in the tissue, strong adhesive interactions are induced, mediated by the expression of integrins. Finally, diapedesis occurs and the activated leukocyte enters the mucosal compartment. Other integrins mediate the classic lesion of inflammatory bowel disease, the crypt abscess.

Potential targets for the treatment of IBD include antagonism of selectins, chemokines or integrins. Interleukin-8 and monocyte chemoattractant protein 1 are potential chemokine targets, while intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and mucosal addressin cellular adhesion molecule 1 (MAdCAM-1) are rational targets, as well. MAdCAM-1, in particular, has the potential added value of tissue specificity, perhaps limiting side effects relating to nonspecific inhibition of integrins and risk of inadequate response to infection.

Injury and repair

Ultimately, injury occurs as a result of many processes and the release of a multitude of directly injurious products, including prostaglandins, leukotrienes, thromboxanes, nitric oxide and other free radicals, and proteases. Direct cellular injury may occur via complement

fixation, antibody dependent cell mediated cytotoxicity, or in other circumstances, by the induction of apoptosis. Injury and repair are dynamic processes, and proper balance between these two processes is critical for normal homeostasis of the intestinal mucosa. The immediate response to injury is epithelial restitution, the process by which epithelial cells spread to cover the denuded area even before mitosis has occurred. Repair follows, with restoration of the normal mucosal architecture, over a longer timeframe. Growth factors, such as transforming growth factor (, epidermal growth factor, keratinocyte growth factor, basic fibroblast growth factor, and platelet derived growth factor may all participate in the process of restitution and repair, and have potential as biological agents in the treatment of IBD. Trefoil proteins are essential to the integrity of the mucoviscous layer that is the first line of defense of the normal gut (22). Trefoil proteins also serve to regulate epithelial growth in the intestine. Trefoil proteins may be another potential biological agent for the treatment of IBD, and might theoretically be delivered orally, by virtue of their marked resistance to proteolytic degradation conferred by their unique structure.

Conclusions

Biological therapies provide unique opportunities to target specific disease processes believed to be important in the pathogenesis of IBD. Contrary to usual methods of broad screenings of libraries of compounds for specific activities, the use of biologic agents permits a more direct means of translating benchtop investigation to the clinic. However, because of the expense of production, and the parenteral delivery systems needed for most biological agents, these therapies must prove to be significantly more effective than compound based therapies. Anti-TNF antibodies have already come into widespread clinical use for Crohn's disease, and are sure to be followed by many other examples of monoclonal antibodies and recombinant cytokines. Nucleic acid-based therapies, such as antisense oligonucleotides and gene therapy, are likely to have additional testing in man in the near future.

References

1. SANDS B.E. Biologic therapy for inflammatory bowel disease. *Inflamm. Bowel Dis.*, 1997, **3** (2) : 95-113.
2. VAN DEVENTER S.J.H., CAMOGLIO L. Monoclonal antibody therapy of inflammatory bowel disease. *Aliment Pharmacol. Ther.*, 1996, **10** (Suppl. 2) : 107-111.
3. ASKARI F.K., MC DONNELL W.M. Antisense-oligonucleotide therapy. *NEJM*, 1996, **334** (5) : 316-318.
4. KRZYMANSKI M, WAAGA AM, ULRICHS K. *et al.* The influence of MHC class II antigen blockade by perfusion with a monclonal antibody on rat renal graft survival. *Transplant International*, 1991, **4** (3) : 180-185.
5. KRUIS W, SCHUTZ E, FRIC P, FIXA B, JUDMAIER G, STOLTE M. Double-blind comparison of an oral Escherichia coli preparation and mesalazine in maintaining remission of ulcerative colitis. *Aliment Pharmacol. Ther.*, 1997, **11** (5) : 853-858.
6. Malchow HA. Crohn's disease and Escherichia coli. A new approach in therapy to maintain remission of colonic Crohn's disease ? *J. Clin. Gastroenterol.*, 1997, **25** (4) : 653-658.
7. REMBACKEN B.J., SNELLING A.M., HAWKEY P.M., CHALMERS D.M., AXON A.T. Non-pathogenic Escherichia coli versus mesalazine for the treatment of ulcerative colitis : a randomised trial. *Lancet*, 1999, **354** (9179) : 635-639.
8. GIONCHETTI P., RIZZELLO F., VENTURI A. *et al.* Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis : a double-blind, placebo-controlled trial. *Gastroenterology*, 2000, **119** (2) : 305-309.
9. GUSLANDI M., MEZZI G., SORGHI M., TESTONI P.A. Saccharomyces boulardii in maintenance treatment of Crohn's disease. *Dig. Dis. Sci.*, 2000, **45** (7) : 1462-1464.
10. CUMMINGS J.H. Dietary carbohydrates and the colonic microflora. *Curr. Opin. Clin. Nutri. Metab. Care*, 1998, **1** (5) : 409-414.
11. JANEWAY C.A., BOTTOMLY K. Signals and signs for lymphocyte responses. *Cell*, 1994, **76** : 275-285.
12. LENSCHOW D.J., SU G.H.T., ZUCKERMAN L.A. *et al.* Expression and functional significance of an additional ligand for CTLA-4. *Proc. Natl. Acad. Sci. USA*, 1993, **90** : 11054-11058.
13. BOUSSIOTIS V.A., FREEMAN G.J., GRIBBEN J.G., DALEY J., GRAY G., NADLER L.M. Activated human B lymphocytes express three CTLA-4 counterreceptors that costimulate T-cell activation. *Proc. Natl. Acad. Sci. USA*, 1993, **90 :** 11059-11063.
14. STÜBER E., STROBER W., NEURATH M. Blocking the CD40L-CD40 interaction in vivo specifically prevents the priming of T helper 1 cells through the inhibition of interleukin 12 secretion. *J. Exp. Med.*, 1996, **183** (2) : 693-698.
15. SHARMA S.D., NAG B., SU X.M. *et al.* Antigen-specific therapy of experimental allergic encephalomyelitis by soluble class II major histocompatibility complex-peptide complexes. *Proc. Natl. Acad. Sci. USA*, 1991, **88** : 11465-11469.
16. ABRAMS J.R., LEBWOHL M.G., GUZZO C.A. *et al.* CTLA4Ig-mediated blockade of T-cell costimulation in patients with psoriasis vulgaris. *J. Clin. Invest.*, 1999, **103** (9) : 1243-1252.
17. DEUSCH K., MAUTHE B., REITER C., RIETHMÜLLER G., CLASSEN M. CD4-antibody treatment of inflammatory bowel disease : one-year follow up. *Gastroenterology*, 1993, **104** (4) : A691.
18. STRONKHORST A., RADEMA S., YONG S.L. *et al.* CD4 antibody treatment in patients with active Crohn's disease : a phase 1 dose finding study. *Gut*, 1997, **40** (3) : 320-327.
19. GROUX H., O'GARRA A., BIGLER M. *et al.* A CD4 sup + T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*, 1997, **389** (6652) : 737-742.
20. FUKAURA H., KENT S.C., PIETRUSEWICZ M.J., KHOURY S.J., WEINER H.L., HAFLER D.A. Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-beta1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients. *J. Clin. Invest.*, 1996, **98** (1) : 70-77.
21. YACYSHYN B.R., LAZAROVITS A., TSAI V., MATEJKO K. Crohn's disease, ulcerative colitis, and normal intestinal lymphocytes express integrins in dissimilar patterns. *Gastroenterology*, 1994, **107** (5) : 1364-1371.
22. SANDS B.E., PODOLSKY D.K. The trefoil peptide family. *Ann. Rev. Physiol.*, 1996, **58** : 253-273.