Hepatitis B virus (HBV), hepatitis C virus (HCV) and hepatitis D virus (HDV) are the leading cause of chronic liver diseases. The aims of the present study are to determine the etiological relationship of HBV and HCV in patients with chronic liver disease in North-Eastern Bulgaria and prevalence of dual and triple infections. A total of 434 patients were investigated for HBsAg, 402 of whom were also tested for anti-HCV. The HBsAg positive subjects were tested for anti-HDV and 32 of them also for HbeAg/anti-Hbe. Separated commercial ELISA kits were used. HBsAg was detected in 132 (30.4%); 10.6% were co-infected with HDV. Anti-HCV was detected in 15.4%. Five of 132 HbsAg positive patients (3.78%) were simultaneously HBV and HCV positive. Two patients out of 132 (1.52%) were positive to HBV, HCV and HDV. Our data indicate that HBV infection was the main cause of chronic liver diseases in North-Eastern Bulgaria, and 10.6% of the patients suffered from severe disease because of co-infection with HDV. HCV plays the same role in 15.4% of the cases. Recently, we observed dually infected (HBV and HCV) and triple infected (HBV, HCV, HDV) patients suffering from severe chronic liver diseases.
Fig. 1. Patients with HBV and/or HCV infection.

Fig. 2. Patients with other HBV markers.
Barcelona, Spain), Hepatitis B surface antigen (ClinPro International, USA). HbeAg/anti-Hbe was also determined using different commercial ELISA test kits: HBeAg & Ab (DIA.PRO, Milano, Italy), ETI-EBK-2 (DiaSorin, Italy), Hepatitis Be Antigen Enzyme Immunoassay (ClinPro International, USA), and Hepatitis Be Antibody (ClinPro International, USA), according to the manufacturer’s recommendations. Anti-HCV was determined using third generation Enzyme Immunoassay (DIA.PRO, Milano, Italy), ETI-AB-HCVK-3 (DiaSorin, Italy), Hepatitis C Virus Enzyme Immunoassay Test (ClinPro International, USA), bioELISA HCV (BIOKIT, Barcelona, Spain), in compliance with the manufacturer’s recommendations. Total serum anti-HDV antibodies were detected, using HDV Ab Enzyme Immunoassay Test Kit (ClinPro International, USA), ETI-AB-DELTAK-2 (DiaSorin, Italy), as per the manufacturer’s recommendations.

Of 434 chronic liver disease patients investigated, HBsAg was detected in 132 (30.4%). HCV was the etiological agent in 15.4% of 402 of these (Fig.1). The data of other Bulgarian studies of patients with cirrhosis (7) shows that 38.5% were HBV positive and 22% anti-HCV positive. The statistically significant differences were proved only for the HBV prevalence (p<0.05). In a similar study by Chatterjee et al the prevalence of HCV infection was about 8% (8). The survey of distribution of these viruses, depending on the age, shows high prevalence of HbsAg in the groups up to 45 years of age (mean 41%). In Moscow, Russia (9), 77% of chronic hepatitis in children was found to be related with HBV infection. In our study there were only 24 children up to 15 years of age investigated, therefore it is difficult to compare our results with statistical data from these authors. The prevalence of anti-HCV (Fig.1) was higher in the patients 16-30 years old (35%) and in those over 60 years old (26%). Relative high anti-HCV prevalence in the older age may be explained by probable transmission of blood and blood products contaminated with HCV in the past. Surprisingly, in our present study, anti-HCV was detected with high prevalence in 16-30 years old patients (35%). High anti-HCV positivity in adolescents was also found in a study by Atanasova et al (10). These findings need further investigation and precise determination of risk factors for acquisition. About 0.4% to 26% of apparently healthy populations in different countries suffer from chronic HCV infection (3, 8, 11).

Fig. 3. Patients with multiple infections.

![Fig. 3. Patients with multiple infections.](image)
patients stratified by age. HBeAg and HBeAb were detected in 40.6% and 31.3% respectively. Most of the patients up to 30 years of age were HBeAg positive. HBeAb were detected more frequently in patients older than 30 years. The detection of HBeAg in HBsAg positive patients is considered as evidence of active viral replication (12). Our data confirmed the current understanding that the age of acquiring of HBV infection affects the course of the disease. In children and adolescents who acquire the infection, there is a long immune tolerance phase with HBeAg positivity. For those, who acquired infection during adolescence or adulthood, the disease progresses directly to the immune clearance phase with seroconversion from HBeAg to HBeAb (13-14). It was observed, as in other studies (15), that severe exacerbations occur with equal frequency in patients who are HBeAg positive and in those with HBeAb.

Over the past few years more complete information about etiological relationship of HBV and HCV with chronic liver diseases has been gathered. Very little data is available regarding coinfections/superinfections with HDV in our region and the interactions between these hepatotropic viruses. Fourteen of 132 (10.6%) HbsAg positive patients were co-infected with HDV, most of them between 16–45 years of age (Fig. 3). The data of another Bulgarian study (7) shows that 6.1% of patients with cirrhosis were co-infected with HDV. Prevalence of HDV in HbsAg carriers varies from 1% to 5% in USA, Western Europe and Asia (16), but is high in those with repeated exposure, such as intravenous drug abusers (20% to 30%) (17). In patients with HDV infection, it has been proved that HDV suppresses HBV. More than 70% of patients superinfected with HDV become chronic carriers of HDV and are at high risk of developing chronic active hepatitis and cirrhosis, and the majority of these die from liver disease (4, 18). Of the patients in this study, only 3 (21%) had laboratory data for active HBV replication (presence of HBeAg in serum).

Five of 132 HbsAg positive patients (3.8%) were simultaneously HBV and HCV positive (Fig. 3) and only 1 of these (20%) was positive for HbeAb. Co-infection with both viruses may occur, because of shared routes of infection. In patients with chronic hepatitis B, the rates of HCV coinfection vary from 9% to 30%, depending on the geographical region (19). Other Bulgarian studies show that 6.6% of the patients with cirrhosis and 4% of chronic HCV infection patients were co-infected with HBV (7, 20). There are no statistical differences when comparing our data with these results. Most of the patients in our study were over 45 years of age. An Italian study shows that dual infections are more common in patients over 50 years of age (21). The exact number of patients infected with both HCV and HBV is unknown, because no large-scale studies have been performed, and also there is a well-described phenomenon of “serologically silent” occult HBV infection (22). A total anti-HBc antibody was not investigated in the present study of chronic liver disease patients. But in our region, there are about 5% of randomly chosen patients with only anti-HBc positivity and HbsAg negative (23). Co-infected patients are often found to have evidence of both HBV and HCV infection, without a clear chronology of infection (12), as was also found in our patients. There are various immune profiles of dually infected patients, and HBV and HCV exert an alternative dominant replication (12, 18, 24-25). There is an inverse relationship between serum HBV replication and serum HCV replication and both viruses have the ability to induce seroconversion of the other (12, 25-26). In our study, among patients with dual infection, only 1 (20%) was Hbe Ag positive.

Two of 132 (1.5%) HbsAg positive patients were anti-HCV and also anti-HDV positive (Fig. 3). In patients with multiple hepatotropic viral infections, the reciprocal influence of each virus remains controversial (19, 25). In most cases HDV acts as the dominant virus (24-25). The interaction between hepatitis viruses in this study was not determined. We assess only HbeAg/HbeAb activity. Concerning liver injury, it has been suggested that patients with dual or triple infection suffer from more severe liver lesions with a higher prevalence of cirrhosis and are at an increased risk for progression to hepatocellular carcinoma (12, 24, 27). Patients with multiple infections should be carefully monitored, and virological assessment is necessary to determine which virus will emerge as a dominant virus, in order to select the most appropriate antiviral treatment.

In summary, the prevalence of HBV and HCV infections varies according to the geographical areas. Patients with chronic viral diseases constitute a reservoir of infected individuals who perpetuate
the infection from generation to generation. We found that nearly 1/3 of chronic liver diseases were related to HBV, and nearly 50% are related to parenterally transmitted hepatitis viruses. The routes and risk factors of acquisition of the infections involved were not identified. The study including a heterogeneity group of patients and all of the routes of infection were possible. The risk factors for acquisition need future investigation, especially in young people. Detection of HBV DNA, HCV RNA and HDV RNA is necessary to be carried out in future investigations. HBV vaccination applied in HCV positive individuals reduces the high risk of dual or triple infections. In Bulgaria, a national expanded program of immunization against HBV has already been started.

REFERENCES


IN VITRO PERMEABILITY OF INTACT AND DE-EPITHELIALISED, HUMAN VAGINAL MUCOSA TO TACROLIMUS AND CYCLOSPORIN A

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Received October 15, 2006 – Accepted February 9, 2007

Immunosuppressants may be applied topically to mucosal surfaces for treating inflammatory diseases, e.g. vulvar lichen planus and Behçet’s disease. The efficacy of the treatment is dependent on the potency of the drug and its penetration into the tissue. In this study the in vitro diffusion characteristics of Tacrolimus (Tac) and Cyclosporin A (CsA) across human vaginal mucosa are investigated and compared. Fresh human vaginal mucosa was snap-frozen in liquid nitrogen and stored at -85 °C. Prior to an experiment, the tissue was defrosted to 20°C in PBS buffer, pH 7.4, and placed in the seven flow cells of a flow-through perfusion apparatus. Either tritiated Tac, or CsA, was then pipetted into the donor chamber of the flow cell. Samples from each flow cell were collected every 2 hours (1.5 ml/h) over a 24-hour period. Statistical analyses were carried out using an F-test. CsA and Tac flux values progressively increased throughout the 24-hour period, and steady state was not reached for either drug. The mean estimated flux values (mean at 16, 20 and 24 h) for Tac (599 ± 44 dpm.cm⁻².min⁻¹) were greater than those for CsA (96 ± 5.0 dpm.cm⁻².min⁻¹). Whole curve comparison indicated statistically significant differences (P = 1.77x10⁻¹⁶³). Both drugs diffuse well across vaginal mucosa. However, mean steady state flux values for Tac were approximately 6 times higher than those for CsA, indicating that human vaginal mucosa is less permeable to CsA than to Tac.

Immunosuppressive drugs are the most predominant and successful form of treatment for autoimmune diseases and prevention of transplant rejection. Most of these drugs act by reducing the production of lymphocytes. Both tacrolimus (Tac), a hydrophobic macrolide (Mw = 822 Da), and cyclosporin A (CsA), a cyclic oligopeptide (Mw = 1202 Da), are calcineurin inhibitors which act by binding to immunophilins (Tac binds to FK binding protein and CsA to cyclophilin), causing inhibition of calcineurin, a calcium/calmodulin-dependent serine/threonine protein phosphatase (1). Calcineurin is integral in regulating the entire immune response involved in graft rejection because of the important role it plays in the proliferation and activation of T-cells by promoting transcription of interleukin-2 (1). In vitro studies, have shown Tac to be 10 -100 times more potent than CsA for inhibiting IL2 production, T-cell proliferation and production of other growth-promoting cytokines (2).

Both Tac and CsA are primarily used systemically for prophylaxis of graft rejection following allogenic organ transplants. However, disorders such as rheumatoid arthritis, uveitis (3), oral lichen planus (4), inverse psoriasis (5) and atopic dermatitis (6-7) have all been successfully treated with Tac. Systemic administration of CsA has also been shown to be an

Key words: permeability, tacrolimus, cyclosporin A, vaginal mucosa
effective form of treatment for atopic dermatitis, psoriasis, pyoderma gangrenosum and other dermatological disorders (8).

Unfortunately, the systemic use of both immunosuppressants may give rise to a wide variety of adverse effects. Topical application of these drugs could therefore be a means to circumvent these adverse effects by providing a more site-specific action. Other advantages of topical drug application include avoiding pre-systemic metabolism in the gastrointestinal tract and hepatic first pass effects. Site-specific immunosuppression with topical CsA (9) and Tac could have extensive clinical applications in the treatment of skin disorders like lichen planus, psoriasis, pyoderma gangrenosum and cutaneous graft-versus-host disease. Additionally, it has also been demonstrated that the use of Tac instead of topical steroids, for the treatment of atopic eczema, does not thin the skin in the way that steroids do, making it a much more suitable alternative for use on facial areas. More recently, topical Tac has been reported to be a successful treatment of genital lichen sclerosis (LS) in three different groups (10).

Several autoimmune or inflammatory conditions of the buccal, or vaginal mucosa are characterised by clinically distinct lesions. The latter are often accompanied by sloughing off of the epithelium, thus rendering the damaged mucosa potentially more permeable to drugs (11). A comparative study of human vaginal and buccal epithelium (12), showed similarities between the two tissue types that support the use of the former as a substitute for the latter mucosa. The similarity in permeability of these two tissue types to a range of permeants has also been demonstrated in various other studies (13-14). In vitro permeability characteristics of drugs through one of these mucosa may therefore be extrapolated to the other with a high degree of confidence.

In view of the potentially useful applications of the topical administration of these two immunosuppressants for a variety of clinical conditions, the aim of the present study is to assess the comparative permeability of intact and de-epithelialised human vaginal mucosa to Tac and CsA.

MATERIALS AND METHODS

Human vaginal mucosa

Tissue specimens were obtained from excess tissue removed from seven postmenopausal women (ages: 60 ± 10 y) subsequent to vaginal hysterectomies at the Louis Leipoldt Hospital, Bellville, South Africa.

Permeability experiments

Prior to the start of each experiment, frozen tissue specimens were thawed to room temperature (20°C) in phosphate buffered saline (PBS, pH 7.4). Thereafter, they were cut into 7 pieces, approximately 4 mm² each, and mounted on small discs to be placed into the flow cells of the flow-through diffusion apparatus (exposed areas 0.039 cm²). Permeation studies were performed on 7 tissue replicates each, for both Tac and CsA. For the Tac experiments, 3.5 µl Tac (1 µCi/µl) was diluted into 3.5 ml PBS; 11.7 µl CsA (1 µCi/µl) was diluted in 3.5 ml PBS for the CsA experiments. These solutions (0.5 ml) were then pipetted into the donor compartment of each flow cell. All radioisotopes were obtained from Amersham Laboratories (Little Chalfont, Amersham, UK). Within minutes, 100 µl aliquots were removed from the 7 donor compartments to determine the donor cell concentration at zero time. For 24 h, PBS at 20°C was pumped at a rate of 1.5 ml/h through the acceptor chambers. Samples were collected every 2 h by means of a fraction collector. For the purposes of this study, the samples at 2, 4, 8, 12, 16, 20 and 24 h were used. To each of these samples, 10 ml of scintillation cocktail (Ready Protein+; Beckman Instruments, Fullerton, CA, USA) was added. The radioactivity was determined using a liquid scintillation counter (Beckman LS 5000TD).

De-epithelialisation

For this study we followed two methods to remove the epithelium from the vaginal mucosa. The first, mechanical de-epithelialisation was performed by using a scalpel to scrape off the uppermost layer from the vaginal mucosa specimens. The second method, heat de-epithelialisation, was carried out as follows: Firstly, a beaker containing distilled water was heated to 80ºC. The thawed vaginal mucosa specimens were then dipped into the beaker for 30s, using tweezers. Upon removal of the tissue from the water, the uppermost layer was peeled off using another pair of tweezers. These de-epithelialised tissue samples were then cut into 7 pieces, 4mm² each, for loading into the donor compartments of the flow-through perfusion apparatus.

Calculation of Flux Values

Flux values (J) across the membranes were calculated using the following relationship J = Q / A x t (dpm x cm⁻² x min⁻¹)

Where Q is the quantity of substance crossing membrane (in dpm); A is the area of the membrane
exposed (in cm²), and t is the time of exposure (in min).

**Steady-State Kinetics**

A specific specimen and tritiated permeant were considered to have reached steady state when no statistically significant differences (P<0.05, t-test) were obtained, over at least 2 consecutive time intervals, between flux values.

**Statistical Analysis**

The GraphPad Prism, Version 4, 2003 computer programme was used to carry out non-linear regression analyses (third order polynomials). To compare flux rate versus time curves, statistical analyses were carried out using an F-test (15).

**Histological Assessment**

Sections of mucosal specimens before (control) and after mechanical as well as heat de-epithelialisation, were subjected to routine histological examination using paraffin-embedded 5-µm sections stained with haematoxylin and eosin.

**RESULTS**

The mean flux values of Tac and CsA across intact, mechanically and heat de-epithelialised human vaginal mucosa, versus time are shown in Figs. 1-3. Flux values for Tac and CsA increased progressively over the 24-hour period for all 10 curves. This indicated that steady state was not reached for either drug in any of the experiments. Mean estimated steady-state flux values were therefore calculated by taking the average of the flux values at 16, 20 and 24 h.

Fig. 1 shows that the mean estimated flux values for Tac (599 ± 44 dpm. cm⁻².min⁻¹) were greater than those for CsA (96 ± 5.0 dpm.cm⁻².min⁻¹). Whole curve comparison indicated statistically significant differences (P = 1.77x10⁻¹⁶).

Mechanical de-epithelialisation of the vaginal mucosa (Fig. 2) resulted in an increased permeability of the mucosa to Tac, but not to CsA. The mean estimated flux value for Tac across mechanically de-epithelialised mucosa was 932 ±137 dpm.cm⁻².min⁻¹, and for Tac across the intact mucosa was 982 ±115 dpm.cm⁻².min⁻¹. For CsA, the mean estimated flux value across mechanically de-epithelialised mucosa was 158 ± 18 dpm.cm⁻².min⁻¹ and across intact mucosa, 152 ± 19 dpm.cm⁻².min⁻¹.

Heat de-epithelialisation of the mucosa (Fig. 3) resulted in the following mean estimated flux values: 863 ± 83 dpm.cm⁻².min⁻¹ for Tac across intact mucosa, 1117 ± 134 dpm.cm⁻².min⁻¹ for Tac across heat de-epithelialised mucosa, 169 ± 18 dpm.cm⁻².min⁻¹ for CsA across intact mucosa, and 185 ± 24 dpm.cm⁻².min⁻¹ for CsA across heat de-epithelialised mucosa, respectively. There was a statistically significant difference between the mean estimated steady-state flux values for Tac; the heat de-epithelialised mucosa showing a greater permeability for Tac. CsA did not show as great a difference, but the heat de-epithelialised mucosae did show slightly greater permeability.

Histological examination of formalin-fixed sections of intact mucosal specimens (Fig. 4a) as well as mucosal specimens after mechanical and heat de-epithelialisation showed that after the latter processes, removal of the epithelium was virtually complete (Figs. 4b and c). Isolated islands of epithelial cells remained attached to the connective tissue (lamina propria), a feature which was more pronounced for the mechanical (Fig. 4b) than for the heat (Fig. 4c) de-epithelialised tissue in the majority of sections examined. Furthermore, an abundance of basal cells was observed for the mechanically de-epithelialised mucosa.

**DISCUSSION**

The advantages of topically administering drugs via routes other than per os are numerous. Mucosal delivery avoids gastrointestinal and hepatic first-pass effects and is, in the case of vaginal and buccal tissue, a convenient site to access allowing prolonged dosing
ratio 1:1.46) than Tac, leading to a corresponding difference in molecular weight between the two drugs. CsA has an almost 50% larger molecular weight (MW difference) than Tac and may be explained as follows.

Human vaginal mucosa was ~6x more permeable to Tac than to CsA and may be explained as follows. The statistically significant differences between the mean estimated steady-state flux values of Tac and CsA across intact human vaginal mucosa showed that human vaginal mucosa was ~6x more permeable to Tac than to CsA and may be explained as follows (Fig. 1). The first factor to consider is the substantial difference in molecular weight between the two drugs. CsA has an almost 50% larger molecular weight (MW ratio 1:1.46) than Tac, leading to a corresponding decrease in permeability of CsA across the mucosa. As mentioned earlier, CsA is an oligopeptide and we can therewith assume that it carries somewhat of a residual positive charge. It will therefore interact favourably with the negatively charged groups in the upper layers of the mucosa, and CsA molecules would have to redistribute from these superficial layers before being able to diffuse into deeper tissue regions.

The graphs depicting the two de-epithelialisation methods show an increase in permeability of the human vaginal mucosa to the two drugs, indicating that damaged mucosa is more permeable than intact mucosa (Figs. 2 and 3). This supports the concept that the main mucosal permeability barrier to drug diffusion is situated in the upper layers of the epithelium (14). Furthermore, this observation has clinical implications in that mucosa, from which the epithelium has sloughed off due to a disease process, will be significantly more permeable to topically applied drugs, potentially enhancing the healing process. The difference in permeability of Tac across heat de-epithelialised mucosa was more pronounced than across the mechanically de-epithelialised tissue. When comparing the effects of the two de-epithelialisation methods on CsA permeability, heat de-epithelialisation also resulted in a greater CsA permeation than mechanical de-epithelialisation. This indicates that heat de-epithelialisation of a mucosal surface is a more efficient method to improve permeability of a substance across that surface. This is most likely due to the fact that heat de-epithelialisation is a more effective means for removing the epithelial layer. When performing this method, one can visibly see the entire epithelial layer peeling off, whereas with mechanical de-epithelialisation, the effect is less easily discernable. Additionally, folds in the mucosal tissue may hinder complete removal of the epithelium (14). Furthermore, this observation has clinical implications in that mucosa, from which the epithelium (14). Furthermore, this observation has clinical implications in that mucosa, from which the epithelium has sloughed off due to a disease process, will be significantly more permeable to topically applied drugs, potentially enhancing the healing process.

No significant increases in the permeability of the vaginal mucosa to CsA were found when tissues were...
Fig. 4a. Photomicrograph demonstrating the histological features of intact human vaginal mucosa (control). The basal cells, the parabasal layer, the stratum spinosum and the superficial layer of the epithelium, as well as the underlying connective tissue layer (lamina propria), are well demonstrated (X 10).

Fig. 4b. Photomicrograph demonstrating the histological features of mechanical de-epithelialised human vaginal mucosa. Islands of epithelial cells are visible attached to the connective tissue (lamina propria). Basal cells are also present (X 10).
mechanically or heat de-epithelialised. This could, once again, be due to the considerable molecular weight of CsA, indicating that even when the mucosal surface is damaged, the CsA molecules are just too large to make any real difference in the penetration characteristics of this compound into the underlying tissues.

Neither of the two permeants reached steady state in the period of time that our studies were performed, i.e. 24 h. We speculate that this is due to the fact that both molecules are predominantly lipophilic. It was demonstrated in a previous study that stacked intercellular lipid lamellae and membrane-bound intracellular granules containing internal lamellae occur within human vaginal mucosa (12). These lamellae are composed of relatively non-reactive lipids; we speculate that it is this lipoidal layer that constitutes the main epithelial permeability barrier to chemical substances through the mucosa.

In conclusion, both CsA and Tac traverse vaginal mucosa well. Considering that most drugs in clinical use possess molecular weights lower than 400 Da, this is a significant finding. Furthermore, we have demonstrated that heat de-epithelialisation is a superior method to mechanical de-epithelialisation for removing the epithelium of the mucosal tissue. Intraleisional penetration of drugs may therefore be enhanced. The superior diffusion characteristics of Tac vs CsA and its higher potency may be clinically significant and translate into a much greater therapeutic efficacy of the former compound, compared to the latter.

ACKNOWLEDGEMENTS

This material is based upon work supported by the South African Medical Research Council. The authors would also like to thank the Stellenbosch University for their financial support. Special thanks are extended to Dr C. le Grange for supplying human vaginal mucosa. The help of Prof. I.O.C. Thompson and Ms J. Coldrey with the histology is gratefully acknowledged.

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TRANSVAGINAL DIFFUSION OF SYNTHETIC PEPTIDES

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Received October 3, 2006 – Accepted January 23, 2007

Topical microbicide peptides are being developed to combat the transfer of HIV, but little is known about the permeation of these compounds through vaginal epithelium. The object of the present study is to investigate the in vitro permeation of synthetic transport peptides through vaginal mucosa. The permeation kinetics of three FITC (fluorescein isothiocyanate)-labelled peptides MEA-5 (Mw = 2911.4 Da), MDY-19 (Mw = 2409.5 Da) and PCI (Mw = 2325 Da) across human vaginal mucosa was studied by means of a continuous flow-through diffusion system. Permeability studies were conducted at concentrations of 1 mM, 0.75 mM and 0.5 mM in PBS buffer at 37°C and 20°C, respectively, and over a time period of 24 h, using fluorospectrophotometry as detection method. Effects of a surfactant on MDY-19 permeation and de-epithelialisation of the vaginal mucosa were also studied. Statistical tests used included an ANOVA and Duncan’s multiple range test to establish steady state diffusion kinetics. All three peptides readily penetrate vaginal mucosa. Microbicides may be coupled to MDY-19 and PCI to be transported transmucosally. Although increased size of the peptide/microbicides complex may decrease mucosal permeability, this could possibly be overcome by the addition of a permeation enhancer, e.g. a surfactant. Removal of the vaginal epithelium increased the flux rates of the peptides across the mucosa and may have implications for a more rapid uptake of these and other microbicides in vivo. Concentration- and temperature- dependency of peptide flux rates must be taken into consideration when performing in vitro permeability studies.

Experience with a variety of compounds has demonstrated that the vagina is a safe and highly efficacious site for drug administration (1-2). However, vaginal administration of drugs still remains a relatively unexplored route for drug administration. Advantages of vaginal administration of drugs include: the administration of lower doses, maintenance of steady drug levels, less frequent administration than with e.g. the oral route, avoidance of the first-pass effect and no effect of gastrointestinal (GI) disturbances on the absorption of the drug (1, 3). This route also allows a woman to self-administer medication continuously for prolonged periods of time.

Currently, a resurgence of interest in peptide and protein drugs exists (4). Many of the latter are endogenous compounds regulating endocrine and other physiological processes in the body (5). These amino acid polymers are increasingly used in major research and development programs, especially due to advances in genetic engineering and biotechnology (6). They may act synergistically with each other and with other agents in the host, e.g. magainin 2 shows synergistic antimicrobial effects with the peptide PGLa.

Due to the development of resistant pathogens, it is important to consider new classes of antibiotics,
such as cationic peptides (5). These small, positively charged peptides are known for their broad-spectrum antimicrobial activity and they are found throughout nature (5). Cationic peptides are produced by most living organisms, from plants and insects to human beings, and form a major part of their immediate defenses against infections. These peptides also have anti-viral and anti-cancer activity as well as the ability to modulate innate immune responses (7).

The unusual physicochemical characteristics of peptides and proteins present considerable challenges to pharmaceutical scientists for their formulation and in selecting a suitable route for their administration. The most commonly used route for protein and peptide delivery has been via parenteral administration due to their lability in the GIT (8). Most proteins and peptides have relatively short half-lives and therefore repeated parenteral administrations (injections) are often required (4). Development of suitable non-parenteral routes for introducing these agents into humans could significantly enhance patient compliance.

Peptides and proteins can be administered topically via mucosal surfaces e.g. vaginal, buccal, nasal and rectal mucosa, thus bypassing first-pass metabolism and making them directly systemically available. Most small peptides, however, do not diffuse readily through mucosal membranes and diffusion enhancers must be added to increase their absorption. Currently much research involves studying the diffusion of small peptide molecules through biological membranes in the presence of chemical permeation enhancers.

This study involves the investigation of diffusion of kinetics of three peptides (MEA-5, MDY-19 and PCI) through human vaginal mucosa. MEA-5 is an antibacterial peptide that binds to cell surfaces, but cannot be internalized. It can act synergistically with other existing microbicides. Both MDY-19 and PCI are transport peptides that bind to cell surfaces and can be internalized. The effects of an absorption enhancer, different permeant concentrations and temperature on the diffusion kinetics of the peptide permeants were also investigated.

MATERIALS AND METHODS

Human vaginal mucosa
Specimens were obtained from excess tissue removed from 43 postmenopausal patients, ages 40-81 years (mean age 58±11 yr SD), following vaginal hysterectomies at the Louis Leipoldt Hospital, Bellville, South Africa.

All surgical specimens obtained were immediately placed in a transport fluid and transferred to our laboratory within 1 h. The transport fluid consisted of a stock solution of Eagle’s Minimum Essential Medium (MEM) without L-glutamine and sodium bicarbonate (Gibco, Paisley, Scotland), to which an antibiotic and antimycotic were added prior to using it for the transport of mucosal specimens. Excess connective and adipose tissue were trimmed away and all specimens were snap-frozen in liquid nitrogen and stored at -85°C for periods up to 6 months, as previously prescribed (9-13). No specimens were obtained where there was clinical evidence of any disease that might have influenced the permeability characteristics of the different specimens. The Ethics Committee of Stellenbosch University and the Tygerberg Academic Hospital approved the study.

Peptides
The three FITC (fluorescein isothiocyanate)-labelled peptides MEA-5 (Mw = 2911.4 Da), MDY-19 (Mw = 2409.5 Da) and PCI (Mw = 2325 Da) were obtained from PEPSCAN, Lelystad, The Netherlands.

Surfactant
The enhancer used was a novel surfactant (prepared in our laboratory). The surfactant suspension contained 13.5 mg/ml DPPC (dipalmitoyl-L-α-phosphatidylcholine) and 1.35mg/ml PG (1,2-dipalmitoyl-L-α-phosphatidylglycerol).

Permeability Experiments
Prior to each permeability experiment, vaginal tissue specimens were thawed at room temperature in phosphate buffered saline (PBS, pH 7.4). The diffusion kinetics of FITC-labelled peptides through thawed frozen vaginal mucosa were then determined. After equilibration of the specimens in PBS, they were carefully cut, so as not to damage the epithelial surfaces, into sections (4 mm in diameter) and then mounted in flow-through diffusion cells (exposed areas 0.039 cm²) with the epithelial surfaces facing upwards. Permeation studies were performed on 7 tissue replicates for each patient. Prior to commencing each permeability experiment, tissue disks were equilibrated for 10 min with PBS (pH 7.4) at 20°C in both the donor and acceptor compartments of the diffusion cells. Following equilibration, the PBS was removed from the donor compartment and replaced with 0.5 ml of either a 1 mM, 0.75 mM or 0.5 mM solution of FITC-labelled peptide in PBS. PBS at 20°C was pumped through the acceptor chambers at a rate of 1.5 ml/h and collected, by means of a fraction collector, at 2 h intervals for 24 h. The permeability study was performed under sink conditions,
i.e. at the completion of each run the concentration of permeant in the acceptor chamber never reached 10% of that in the donor compartment. For the detection of FITC peptides, fluorospectrophotometry (emission: 520 nm and excitation: 497 nm) was carried out using a Perkin-Elmer spectrophotometer (Perkin-Elmer, MA, USA). Experiments were also conducted at 37°C.

Mechanical and heat stripping

Mucosal surfaces were de-epithelialised by a heating method, as well as mechanically, to mimic ulceration and its effects on passage of the peptides. De-epithelialisation by means of heat involved the submerging of the vaginal tissue at 80°C water for 30 s, thereafter removing the epithelial layer with tweezers. Mechanical de-epithelialisation was conducted by carefully scraping off the epithelium from the vaginal mucosa with a scalpel, without damaging the underlying connective tissue layer.

Calculation of Flux Values

Flux (J) values of the various chemical compounds across the vaginal membranes were calculated by means of the relationship: \( J = Q/A \times t \), where \( Q \) = quantity of compound crossing membrane (pmoles), \( A \) = membrane area exposed (cm²) and \( t \) = time of exposure (min).

Steady-State Kinetics

Steady state was assumed to have been reached for a particular specimen and chemical compound when no statistically significant differences (p<0.05) at the 5 % level (t-test with Welch’s correction) between flux values were obtained over at least 2 consecutive time intervals.

**Fig. 1.** The overall mean flux values at 20°C of MEA-5 and MDY-19 versus time across vaginal tissue.

**Fig. 2.** The overall mean flux values at 20°C of MDY-19 versus time across vaginal tissue with and without surfactant as a penetration enhancer.

**Fig. 3.** Comparison of the flux ratios (de-epithelialised/control) at 20°C obtained for MEA-5 versus time across human vaginal mucosa after heat and mechanical de-epithelialisation.

**Fig. 4.** Overall mean flux values at 37°C of MDY-19 versus time across intact vaginal mucosa and de-epithelialised mucosa by means of heat.
Non-linear regression analyses (third order polynomials) were performed using a GraphPad Prism, version 4, 2003 computer programme. An F-test was used to compare entire curves (14). A t-test at steady state was also performed for comparative purposes. A significance level of 5% was used for all tests and comparisons.

RESULTS

The overall mean flux values of MEA-5 and MDY-19 versus time across vaginal tissue (20°C) are shown in Fig. 1. Both MEA-5 and MDY-19 permeated vaginal mucosa well. MDY-19 had a higher flux rate than MEA-5, commensurate with its smaller molecular size (weight). MEA-5 and MDY-19 reached steady state between 10 and 12 hours.

The overall mean flux values of MDY-19 across vaginal tissue (20°C) with and without surfactant as a penetration enhancer are shown in Fig. 2. The surfactant enhanced the flux rate of MDY-19 approximately 1½ times (hours 4-12). The surfactant decreased the lag time of the peptide. The control group reached steady state after 10-12 hours, and the group with the surfactant enhancer had a lag time of 8-10 hours.

Vaginal mucosal surfaces were de-epithelialised by means of mechanical and heat stripping. The comparison of the flux ratios (de-epithelialised/control) obtained for MEA-5 across human vaginal mucosa after heat and mechanical de-epithelialisation is shown in Fig. 3. Both heat and mechanical de-epithelialisation of mucosa significantly enhanced flux values of MEA-5 (2.5 x) compared with controls (20°C) (Fig. 3), although no marked advantages could be discerned between the two methods. Precipitation of MEA-5 was noted with the initial experiments; therefore further permeability studies were conducted at 37°C. The overall mean flux values of MDY-19 and PCI across intact vaginal tissue (control group) versus de-epithelialised tissue are respectively shown in Figs. 4 and 5. De-epithelialisation of mucosa significantly enhanced flux values of MDY-19 (1.5 x) and PCI (1.2 x) compared with controls (37°C).

The mean steady state flux values of MDY-19 and PCI compared at different concentrations and temperatures (20 °C and 37°C) are shown in Fig. 6 (certain error bars are not visible due to the minimal variations in mean steady state flux values). The mean steady state values of MDY-19 increased with an increase in concentration, but the mean steady state flux values of PCI increased from 0.5 to 0.75 mM and then decreased again as the concentration of PCI was increased to 1 mM. The mean flux at values of 1 mM MDY-19 and 0.75 mM PCI were the highest.

DISCUSSION

HIV is spreading rapidly, especially in sub-Saharan Africa and Southeast-Asia. New prophylactic strategies, e.g. the use of microbicidal vaginal formulations, which have the advantage that women can take control of their own safety, are being investigated (15). Cationic peptides seem to be promising candidates as new therapeutic agents, because they have activity against malaria parasites, and viruses, including HIV, HSV, influenza A virus and vesicular stomatitis virus (7). Where antibiotics only have activity against bacteria, cationic peptides have a wide range of activities against bacteria, fungi, enveloped viruses and eukaryotic parasites.

Both MEA-5 and MDY-19 readily penetrate vaginal mucosa. Novel microbicides may be coupled to the transport peptide MDY-19 to be transported transmucosally or into cells. Although the increased size of a MDY-19/microbicide complex may decrease mucosal permeability, this could possibly be offset by the addition of an appropriate surfactant-containing permeation enhancer. The surfactant used in this study enhanced the flux rate of MDY-19 between 8-12 hr approximately 1 ½-fold (Fig. 2).

De-epithelialisation mimics the situation when integrity of vaginal epithelium is compromised due
to disease or local trauma and significantly increased the permeability of MEA-5, MDY-19 and PCI over controls by approximately 2.5x, 1.5x and 1.2x, respectively. This supports the premise that the main barrier is located in the epithelium and it may have possible implications for increased in vivo uptake of microbicides as well as HIV by traumatised vaginal mucosa. It is in keeping with the finding that other sexually transmitted diseases e.g. genital herpes, chlamydial infection, trichomoniasis (ulcerative and non-ulcerative) are known to cause disruption of the vaginal epithelium and can increase HIV transmission 3- to 10-fold (16-17). Initially two methods e.g. mechanical and heat de-epithelialisation were used to establish the most effective and convenient method to remove epithelium from the vaginal mucosa. The MEA-5 peptide was used to evaluate these two de-epithelialisation methods. Because de-epithelialisation by heat appeared to be the more convenient method, it was therefore used for mucosal de-epithelialisation for the experiments using the peptides MDY-19 and PCI.

Precipitation of the peptides MDY-19 and PCI on the vaginal mucosa during the permeability experiments at room temperature (20°C) and at a concentration of 1 mM occurred. For this reason, permeability studies of MDY-19 and PCI were repeated at concentrations of 1 mM as well as lower concentrations of 0.75 mM and 0.5 mM, in PBS buffer, at a temperature of 37°C. The overall mean flux values of 1 mM MDY-19 and PCI at 37°C at steady state were higher than the overall mean flux values at 20°C. At temperatures of 37°C the mean flux values at steady state of MDY-19 increased with concentration according to well-established diffusion theory. However, the flux values of PCI increased from 0.5 to 0.75 mM and then decreased again as the concentration of PCI was increased to 1 mM (Fig. 6). This may indicate a decrease in solubility of the latter peptide. PCI has a less overall positive charge than MDY-19 since it contains less of the basic amino acid, arginine. At high concentrations (1 mM) precipitation of PCI may occur, because it is less water-soluble than MDY-19 and therefore the flux values of the former peptide across vaginal tissue decrease. At room temperature, the mean steady state flux values for MDY-19 are 1.9x higher, and at 37°C it is 1.03x higher than the corresponding values found for PCI. This may be explained by a higher water solubility of PCI at a higher temperatures, leading to less precipitation on the vaginal tissue, which in turn yields higher flux values.

The average flux values at steady state of 1 mM MDY-19 and 0.75 mM PCI were the highest, and

Fig. 6. The mean steady state flux values obtained for various concentrations of MDY-19 and PCI across intact vaginal mucosa at 20°C and 37°C.
it would therefore seem that these concentrations are the most suitable for conducting permeability experiments.

In conclusion, we have demonstrated that the de-epithelialisation of the vaginal mucosa increased the permeability of all three peptides tested in the study. This supports the premise that the main epithelial barrier is located in the epithelium and it may have possible implications for increased microbicidal as well as HIV uptake in vivo. Furthermore, the results of the study have demonstrated the concentration- and temperature dependency of flux rates of peptides across vaginal mucosa. This should be taken into consideration for determining optimum diffusion conditions for in vitro permeability studies.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial assistance of the SA Medical Research Council, the Harry Crossley Fund, EMPRO and Pepscan. We also thank Dr C. le Grange for supplying the vaginal specimens.

DISCLAIMER: Any opinion, findings and conclusions or recommendations expressed in this material are those of author(s) and therefore the MRC does not accept any liability in regard thereto.

REFERENCES

ORAL ALLERGIC SYNDROME AND RECOMBINANT ALLERGENS
rBet v 1 AND rBet v 2

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Received March 30, 2006 – Accepted January 18, 2007

IgE cross-reactivity to Recombinant Allergens (RA) rBet v 1 and rBet v 2 (profilin) and food allergens may represent a basis for the development of oral allergic syndrome (OAS). We performed a retrospective study on 59 patients polisensitized to pollens and food allergens. They were given an assay of specific IgE against RA and, when positive, we calculated the percentage of these subjects who presented an OAS. As a result, 21 out of 59 patients (35.6%) were positive to rBet v 1, 23 out of 59 (38.9%) to profilin, and 5 out of 59 (8.4%) to both. Among RA positive patients 7 (33.3%) with specific IgE against rBet v 1 presented OAS, and 8 (34.7%) suffered from OAS. IgE to peanut and apple were mostly represented (57.1%) in patients positive to rBet v 1, while in subjects positive to profilin, we mainly observed IgE against peanut (75.0%). We suggest the importance of evaluating hypersensitivity to RA to predict the increase of OAS and, in particular, to reveal which fruits could be associated to OAS.

Type I allergies are immunological disorders that afflict a quarter of the world’s population. Although not severely invalidating, they are a relevant health problem because of their increasingly high prevalence. Consequently, there is a growing need for accurate in vitro diagnosis by means of purified, well-defined and standardized allergenic molecules, based on the use of recombinant allergens. In the last decade recombinant DNA techniques were applied to produce such proteins with immunological characteristics identical to the naturally occurring proteins (1) and they have been used to isolate and characterize many allergens. The main research field focused on: 1) characterization of allergens; 2) purification from pollen or food extracts and cloning of allergen genes. Bet v 1, the major allergen of birch pollen, was the first plant allergen to be cloned, sequenced and expressed in Escherichia coli (2-3). Using a murine model of type I allergy, mice immunized with crude leaf extracts containing Bet v 1 with purified rBet v 1 produced in E. coli or with birch pollen extract, generated comparable allergen-specific IgE and IgG1 antibody responses and type I skin test reactions. These results demonstrate that non-purified Bet v 1 retains the same immunogenicity as purified Bet v 1 produced in E. coli. (4). A large body of evidence has also clearly shown that RA have an immunological behaviour similar to their native counterpart when analysed by Western blot, skin prick test and histamine release assay. In this light, the use of RA may represent an improvement for in vitro diagnosis of the allergic disease. According to this view, since September 2004 we commonly use in our laboratory RA as rBet

Key words: oral allergic syndrome, recombinant allergens
v 1 and rBet v 2 for IgE specific assays, especially to further evaluate patients showing polisensitization to pollens (particularly birch) and food at the prick test. In this case, because of their cross-reactivity among different inhalant and vegetable species, recombinant allergens may represent a useful tool to better understand which allergen is responsible for the allergic reaction. Once established the relevant allergen, a specific immunotherapy can be proposed to polisensitized patients who could not otherwise receive this treatment. Bet v 1 shows homologies to various food allergens, such as hazelnut, apple, celery and tomato. Allergy to Bet v 2 was also described in 10–15% of patients sensitised to birch pollen (5). IgE cross reactivity between pollen and food allergens represents the molecular basis for the oral allergy syndrome (OAS), a manifestation of food allergy (6) affecting the lips, mouth and pharynx. OAS normally occurs in people with asthma or hay fever from pollen allergies when eating fresh fruits or vegetables. It can also affect people who are allergic to shell fish and eggs. Adults appear to be more affected than children. An allergic response occurs when the activated immune system cannot distinguish the difference between pollen proteins and food proteins and symptoms develop when the immune system recognizes a “cross reactive” protein. Rapid onset of itching of the lips, mouth or pharynx, and swelling of the lips, tongue, throat and palate are the most common symptoms of OAS. Other symptoms may include gingivitis, conjunctivitis or rhinitis. In rare cases, asthma or anaphylaxis may be triggered. Symptoms normally appear within minutes after eating the offending food. Individuals with birch allergy may cross react with apples, pears, almonds, peaches, apricots, cherries, plums, prunes, kiwis, carrots, celery, fennel, parsley, coriander, peppers and potatoes, as with hazelnuts, almonds and walnuts. Grass pollen allergies may crossreact with melons, tomatoes and oranges. Mugwort pollen allergy is associated with celery, fennel, carrots, parsley, coriander, sunflower and peppers. In this study we analysed patients with symptoms of respiratory and/or food allergy and with a prick test revealing a polisensitization to pollens and foods. An assay of serum specific IgE against the most common inhalant and food allergens was performed for each enrolled subject; in patients with specific IgE, especially if polysensitized, we tested IgE-specific for rBet v 1 and rBet v 2. Finally, the percentage of RA-positive patients with an OAS was evaluated to confirm literature data on the role of such allergens as predictors of OAS and to suggest specific IgE for rBet v 1 and rBet v 2 as a first-line in vitro test for OAS diagnosis, replacing an expensive and extended analysis of all the cross-reacting pollens and food allergens.

MATERIALS AND METHODS

We performed a retrospective study on 59 patients (mean age 29.8 yrs; age range 6–56 yrs) from September 2004 to April 2005. No patients had received specific immunotherapy before the study. Each patient had a history of allergic reactions to pollen (asthma, rhinitis, rhino-conjunctivitis) or food (urticaria, OAS), with a positive in vivo skin prick test to inhalant and food allergens and, after a serum assay of specific IgE (CAP system; Phadia, Uppsala, Sweden), was positive to a great variety of inhalant and/or food allergens, as confirmed by the prick test. After this first series of analysis, an assay of specific IgE against rBet v 1 and rBet v 2 (CAP system; Phadia, Uppsala, Sweden) was performed on all patients to assert to what these subjects were actually allergic, that is, if we were observing a true polysensitisation or a sensitisation only to the allergen in common to plants and foods. We then calculated the number of patients with specific IgE against RA, also manifesting OAS, and the percentage of fruits mainly implicated in this clinical manifestation.

RESULTS

Twenty-one out of all the 59 polysensitized patients (35.6%) resulted allergic to rBet v 1, while 23 patients (38.9%) were positive to rBet v 2. Only 5 subjects were positive to both RA. All subjects allergic to rBet v 1 and/or to rBet v 2 were also positive to birch. As a clinical manifestation, twenty-one out of all 59 patients (35.6%) presented only OAS without respiratory symptoms, while the others (38.9%) showed a variety of symptoms, such as respiratory ones, urticaria, and abdominal disturbance. Seven subjects vs. 21 (33.3%) with specific IgE to rBet v 1 presented OAS, and 8 patients positive to rBet v 2 showed an OAS (34.7%). The mean age of the patients with OAS was 30.5 yrs. Subjects with OAS and specific IgE to RA did not present any respiratory
symptoms. The average concentration of specific IgE in patients with OAS positive to rBet v 1 was 11.2 KUA/l (III class), while for rBet v 2 we observed a mean concentration of 3.10 KUA/l (II class). The mean concentration value for birch was 9.67 KUA/l (III class). A number of subjects positive to rBet v 1 and rBet v 2 presented only respiratory symptoms. In particular, with regard to rBet v 1, 2 vs. 21 (9.5%) had asthma and rhinoconjunctivitis, 2 vs. 21 (9.5%) had only asthma, and 3 vs. 21 (14.2%) showed only rhinoconjunctivitis. Instead, patients allergic to rBet v 2 presented asthma and rhinoconjunctivitis (1 vs. 23, 4.3%), 2 vs. 23 (8.6%) only asthma, 5 vs. 23 (21.7%) only rhinoconjunctivitis, and finally one patient (4.3%) had urticaria. We also evaluated food allergy in patients with OAS and RA positive IgE to search for the specific food allergen associated to OAS (Tables I). As a result, we found that peanut and apple probably caused the greatest number of OAS symptoms in patients positive to rBet v 1, while peanut was the most involved food allergen in rBet v 2 positive OAS patients (Table II).

**DISCUSSION**

Many atopic patients also presented a food allergy as a consequence of cross reactivity between inhalant and food allergens. One of the most important and immediate food-reaction allergies

<table>
<thead>
<tr>
<th>Patients</th>
<th>rBet v 1</th>
<th>rBet v 2</th>
<th>Allergens</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.80</td>
<td>&lt;0.35</td>
<td>Hevea brasiliensis, Poa pratensis, Parietaria judaica, Betula verrucosa, <em>Amigdalus communis</em></td>
</tr>
<tr>
<td>2</td>
<td>&lt;0.35</td>
<td>1.75</td>
<td>Betula verrucosa, Olea europea, Artemisia vulgaris, Poa pratensis, <em>Triticum aestivum, Arachis hypogeae</em></td>
</tr>
<tr>
<td>3</td>
<td>0.38</td>
<td>&lt;0.35</td>
<td>Betula verrucosa, Prunus persica, <em>Arachis hypogeae, Malus sylvestris, Hevea brasiliensis</em></td>
</tr>
<tr>
<td>4</td>
<td>1.41</td>
<td>&lt;0.35</td>
<td>Poa pratensis, Artemisia vulgaris, Betula verrucosa, <em>Arachis hypogeae, Malus sylvestris</em></td>
</tr>
<tr>
<td>5</td>
<td>16.4</td>
<td>&lt;0.35</td>
<td>Betula verrucosa, <em>Arachis hypogeae, Corylus avellana, Malus sylvestris, Pyrus communis</em></td>
</tr>
<tr>
<td>6</td>
<td>4.39</td>
<td>&lt;0.35</td>
<td>Phleum pratensis, Betula verrucosa, <em>Prunus persica</em></td>
</tr>
<tr>
<td>7</td>
<td>6.13</td>
<td>1.06</td>
<td>Betula verrucosa, Olea europea, Parietaria judaica, <em>Arachis hypogeae, Corylus avellana, Prunus armeniaca, Malus sylvestris, Juglans spp, Prunus persica</em></td>
</tr>
<tr>
<td>8</td>
<td>&lt;0.35</td>
<td>9.24</td>
<td>Hevea brasiliensis, Betula verrucosa, <em>Arachis hypogeae</em></td>
</tr>
<tr>
<td>9</td>
<td>&lt;0.35</td>
<td>4.63</td>
<td>Hevea brasiliensis, Dermatophagoides pteronyssinus, Betula verrucosa, <em>Arachis hypogeae, Prunus persica</em></td>
</tr>
<tr>
<td>10</td>
<td>&lt;0.35</td>
<td>0.38</td>
<td>Betula verrucosa, Phleum pratensis, Olea europea, <em>Prunus armeniaca</em></td>
</tr>
<tr>
<td>11</td>
<td>&lt;0.35</td>
<td>0.53</td>
<td>Betula verrucosa, Olea europea, Cupressus sempervirens, <em>Arachis hypogeae, Prunus persica, Malus sylvestris, Corylus avellana, Malus sylvestris, Pyrus communis</em></td>
</tr>
<tr>
<td>12</td>
<td>43.2</td>
<td>0.46</td>
<td>Phleum pratensis, Betula verrucosa, <em>Juglans spp, Hevea brasiliensis</em></td>
</tr>
<tr>
<td>13</td>
<td>&lt;0.35</td>
<td>6.78</td>
<td>Poa pratensis, Betula verrucosa, <em>Juglans spp, Arachis hypogeae, Prunus persica</em></td>
</tr>
</tbody>
</table>

**Table I.** Food allergens associated with OAS. Concentration of specific IgE are expressed in KUA/l. Also inhalant allergens are indicated.
is OAS, and it has been suggested that in patients with OAS pollen allergens may be responsible for the elicitation and the maintenance of disease (7). In particular, specific IgE against apple has been considered the only predictive factor for the onset of OAS symptoms (8). We performed a retrospective study in patients previously diagnosed for birch allergy also showing multiple sensitisations to cross-reacting inhalant allergens; all subjects received a serum assay with the fluoroenzyme immunoassay technique to the major birch allergen, rBet v 1, and another important allergen cross-reacting with food components, that is rBet v 2. We use these allergens in their recombinant form because of their purity and perfect correspondence with their native form, as already confirmed by many studies in the literature. We focused on Bet v 1 and Bet v 2, because they can be considered as panallergens, since they are found in a great number of plant and food species.

We found that many patients with specific IgE against inhalants, in particular birch, and food allergens, were also positive to RA (35.6% for rBet v 1 and 38.0% for rBet v 2). We also observed that, of the 21 subjects with specific IgE to rBet v 1, also positive to birch, a significant percentage of subjects presented an OAS as clinical manifestation, and that 8 vs. 23 patients positive to profilin (34.7%) showed OAS symptoms. The mean age of these patients (30.5 yrs), confirms that this disease mainly affects adults. Our data seem to be in agreement with the findings of De Amici et al. (6), who reported OAS symptoms in 12 out of 33 (36%) selected patients with skin prick test positive to birch pollen. In the same study, considering the patients positive to the two RA of birch, the authors presented an OAS frequency of 35% for patients positive to rBet v 1 and of 50% in those positive to rBet v 2. For the two patients contemporarily positive to RA, we found a 100% correspondence between the presence of specific IgE and OAS, and although a preliminary finding due to the limited number of subjects, it could suggest a higher probability of manifesting OAS in the presence of specific IgE to both antigens. With regard to the meaning of specific IgE against apple, considering only the predictive factor for the appearance of OAS symptoms, we found that the highest percentage of subjects positive to apple, as shown in Table II, was 57.1% (4/7) for rBet v 1 and 25.0% (2/8) for rBet v 2. Even in this case, despite the limited number of patients, we could assert, especially for rBet v 1, for which IgE specific against apple were the most represented, that our data agree with the findings of De Amici et al. (8). Instead, with regard to rBet v 2, we may state that the risk fruits for the development of an OAS are peanuts (75%; 6/8 patients), and that also peach (50%; 4/8 subjects) can be considered a risk fruit for OAS. Also in the case of rBet v 1, peach (42.8%; 3/7 patients) seems to be an important fruit for the development of OAS. Finally, the mean concentration of serum specific IgE against RA in patients with OAS was higher for rBet v 1 (class III) than for rBet v 2 (class II), suggesting a greater capability of rBet v 1 to induce OAS. As a whole, in our study we show the importance of the use of RA to predict the increased food symptomatology, such as OAS in patients

<table>
<thead>
<tr>
<th>Allergens</th>
<th>rBet v 1 (n = 7)</th>
<th>rBet v 2 (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amigdalus comm. (almond)</td>
<td>1 (14.2 %)</td>
<td>1 (12.5 %)</td>
</tr>
<tr>
<td>Triticum aestivum (wheat)</td>
<td>0</td>
<td>1 (12.5 %)</td>
</tr>
<tr>
<td>Prunus persica (peach)</td>
<td>3 (42.8 %)</td>
<td>4 (50.0 %)</td>
</tr>
<tr>
<td>Arachis hypogaeae (peanut)</td>
<td>4 (57.1 %)</td>
<td>6 (75.0 %)</td>
</tr>
<tr>
<td>Malus sylvestris (apple)</td>
<td>4 (57.1 %)</td>
<td>2 (25.0 %)</td>
</tr>
<tr>
<td>Pyrus communis (pear)</td>
<td>1 (14.2 %)</td>
<td>1 (12.5 %)</td>
</tr>
<tr>
<td>Corylus avellana (hazelnut)</td>
<td>2 (28.5 %)</td>
<td>2 (25.0 %)</td>
</tr>
<tr>
<td>Prunus armeniaca (apricot)</td>
<td>1 (14.2 %)</td>
<td>2 (25.0 %)</td>
</tr>
<tr>
<td>Juglans spp (walnut)</td>
<td>2 (28.5 %)</td>
<td>3 (37.5 %)</td>
</tr>
</tbody>
</table>
allergic to birch and in particular also sensitised to its major allergen (rBet v 1) and rBet v 2. We suggest that the use of these tools may open the doors to a new field of research in allergology, and that it could be considered as a new modality to approach specific immunotherapy in polysensitized patients.

REFERENCES


ALLERGIC SUBJECTS HAVE MORE NUMEROUS RESPIRATORY INFECTIONS AND SEVERE GASTROINTESTINAL INFECTIONS THAN NON-ALLERGIC SUBJECTS: PRELIMINARY RESULTS

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Received July 17, 2006 – Accepted January 18, 2007

Allergic disorders are characterized by Th2-polarization, thus physiological Th1-dependent mechanisms for fighting infectious diseases (ID) may be defective. This study aims at evaluating the number and duration of respiratory (RI) and gastrointestinal diseases (GI) in allergic and non-allergic subjects in a particular community: the crew of an operative warship consisting of 189 persons (171 males and 18 females). The study period lasted for 5 months during an operative mission. The number and duration (days) of RI and GI were evaluated. Allergic subjects experienced more numerous RI (p=0.0029) and more severe GI (p=0.0001) than non-allergic subjects. This preliminary study provides evidence that allergic subjects may have more numerous RI and prolonged GI than non-allergic subjects.

In addition, treatments with drugs able to reduce ICAM1 expression have diminished both the number and severity of RI in allergic children (6).

Infectious diseases (ID) of respiratory (RI) and gastrointestinal (GI) system are frequent and constitute a demanding challenge for the physician (7). Moreover, ID represents a social problem both concerning the pharma-economy and the impact on the social milieu of the subject, mainly during journeys or heavy work. Recently, it was evidenced that allergic children have more numerous and severe RI than non-allergic children (8). However, there is no data concerning the different impact of RI and GI on allergic and non-allergic adults. Therefore, this preliminary study was performed to evaluate the number and duration of RI and GI in allergic and non-allergic subjects living in a particular community: an operative warship.

Key words: allergy, respiratory infections, gastrointestinal infections, Th1 and Th2 cells
MATERIALS AND METHODS

Study design

The complete crew of an operative warship (a frigate of the Italian Navy) was enrolled in the study: 189 persons (171 males and 18 females). It is to note that Navy personnel is continuously checked and trained. Moreover, asthma diagnosis is incompatible with the service. Subjects were subdivided into two groups according to the presence of allergy (i.e. allergic rhinitis). Allergic rhinitis was diagnosed on the basis of positive history of nasal-ocular symptoms and skin prick test (9). This model was chosen as a selected group of subjects allows to obtain more precise information. Indeed, the population consists of normal and allergic subject. All of them live in the same restricted environment (the ship), therefore all of them are exposed to the same harmful agents. In addition, the crew is under 24 hour control of a physician. The study was performed during two operative missions lasting 5 months in 2005. The period of operative mission was considered as it is easier to evaluate subjects when confined in a restricted area. Moreover, favourable conditions of contracting infections may occur during this stressing situation.

All the visits to the infirmary were recorded. The diagnosis of RI was made when at least 2 symptoms or fever (auxiliary temperature ≥38°C) were present for at least 48 hours. The symptoms taken into consideration for diagnostic purposes were: mucopurulent rhinorrhea (stuffy and/or dripping nose), sore throat, cough (dry or productive), otalgia (earache), fever, dyspnoea, and mucopurulent secretion.

The diagnosis of GI was made when one or more digestive symptoms were present: nausea, vomiting, diarrhoea, and colic.

The primary end-point was the number of RI and GI episodes recorded during the period study in the two groups. The secondary outcome was the total duration of RI and GI episodes (days). RI were considered as mild or severe. Mild RI involved the rhinopharynx (rhinitis, rhinopharyngitis with possible involvement of tonsils) and/or the larynx. Severe RI involved the middle ear, the lower airways or the paranasal sinuses.

Statistical analysis

The main descriptive statistics were calculated for each variable: mean, median and standard deviation for quantitative variables, absolute and relative frequencies for qualitative variables. Also the 95% confidence limit was calculated whenever relevant. The analysis was carried out using the $\chi^2$ test.

RESULTS

Population

189 subjects (the complete crew) were studied: age 25.9 ± 7.12 years, 171 males and 18 females. All subjects were healthy and without significant disorders, as they are continuously checked. Allergic rhinitis was diagnosed in 52 subjects. Allergic subjects were mainly sensitized to: house dust mites (29), grasses (19), olive tree (13), Parietaria (13), cat (7), dog (4), hazel (4), and birch (4). Globally, 133 visits were performed: 55.7% for traumatic events, naupatia, or dermatologic disorders.

Table I. Mean ± SD number and duration of respiratory infections (RI) and gastrointestinal infections (GI) in controls (such as non-allergic subjects) and allergic subjects.

<table>
<thead>
<tr>
<th>Total number and forms of ID</th>
<th>Controls (n=88)</th>
<th>Allergic subjects (n=45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RI number (p = 0.0029)</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>Total RI duration (days) (p=n.s.)</td>
<td>4.1 ± 2.3</td>
<td>4.6 ± 1.8</td>
</tr>
<tr>
<td>Total GI number (p=n.s.)</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>Total GI duration (days) (p = 0.0001)</td>
<td>2.7 ± 1.3</td>
<td>6.5 ± 3.7</td>
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</table>
24.8% of the visits were for RI, 19.5% was for GI.

**Primary outcome**

Allergic subjects showed a significantly higher number (18) of RI in comparison with non-allergic group (15) (p=0.0029) (Table I).

All RI were mild and self-resolving: 8 rhinopharyngitis, 7 rhinitis, and 3 rhinosinusitis in allergic group; 7 rhinopharyngitis, 7 rhinitis, and 1 rhinosinusitis in normal subjects.

The number of GI was not significantly different in the two groups: 18 episodes in normal subjects and 8 in atopic subjects. All GI were mild gastroenteritis and self-resolving.

**Secondary outcome**

The duration of RI was slightly longer in allergic subjects (4.6 days) than in normal subjects (4.1). On the other hand, allergic subjects showed longer duration of GI (6.5 days) in comparison with non-allergic group (2.7 days) (p=0.0001) (Table I).

**DISCUSSION**

This preliminary study evidences that allergic subjects have more numerous RI and prolonged GI than non-allergic subjects. These findings confirm a previous report (8) providing evidence that allergic children have more numerous and severe RI than non-allergic children. Allergic subjects may have more infections than normal subjects as allergic reaction is characterized by mucosal inflammation that predispose to infections. In addition, allergic subjects express ICAM1, adhesion molecule involved in inflammatory events as ligand for LFA1 expressed on leukocytes, but also receptor for rhinovirus (5). These findings may be reinforced by the concept that anti-allergic treatments may reduce the number and severity of RI (6). Moreover, data concerning GI are very interesting as this represents the first evidence on this issue. A possible explanation may be that allergy per se may induce reduced immune defences. Indeed, allergic subjects present a typical Th2-polarization with consequently reduced Th1-response that adequately fights infections mainly by IFNγ.

On the other hand, these results have to be considered as preliminary, as further studies should be conducted to verify this hypothesis. More consistent numbers of subjects and longer periods of observation must be considered. The present study was performed with a selected group of subjects during a relatively short period. In spite of these limits, the present findings underline the importance of the type of immune pattern, such as the physiologic Th1-polarization or the allergic Th2-polarization, as the crucial factor involved in the response to infectious diseases. In addition, the particular condition of carrying out the study during operative missions allows to better investigate the different response to infections in allergic and non-allergic subjects.

In conclusion, this preliminary study provides the information that allergic subjects have more numerous and severe ID than non-allergic subjects, and this result might suggest that allergy could have a negative role in both RI and GI, although a further case control study is needed to investigate more accurately the cause-effect role of allergy in infections.

**REFERENCES**


SHORT REPORT

FRONTAL FIBROSING ALOPECIA IN A PREMENOPAUSAL WOMAN: IMMUNOHISTOCHEMICAL AND IMMUNOFLUORESCENCE ANALYSIS

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Received September 29, 2006 – Accepted January 29, 2007

Frontal fibrosing alopecia (FFA) was first described in 1994 and is considered a variant of lichen planopilaris in a patterned distribution that primarily affects postmenopausal women. Cicatricial alopecia of the frontoparietal hairline is characteristic; the affected area appears as a shiny, band-like zone of incomplete hair loss with skin-coloured small hyperkeratotic papules. We describe a case of a premenopausal woman with this rare disease. To date there are very few reports of FFA in premenopausal women. The immunohistochemical investigation with lymphocytic markers (CD4, CD8, CD20, CD45RA, CD57) revealed CD8 positive lymphocytes penetrating the follicular epithelium. Direct immunofluorescence showed clumped deposition of IgM along the follicular basement membrane zone.

Frontal fibrosing alopecia (FFA) is a progressive scarring alopecia. FFA was first described by Kossard in 1994 (1) and is considered a variant of lichen planopilaris. Elderly postmenopausal women are typically affected, and present with pruritic, cicatricial alopecia of the frontotemporal hairline. The affected area appears as a shiny, band-like zone of incomplete hair loss that is of variable width (1-8 cm), and hairs with perifollicular erythema and hyperkeratosis (2). We describe a case of a premenopausal woman with this rare disease. To date there are very few reports of this disease in premenopausal women.

MATERIALS AND METHODS

A 36-year-old Caucasian, Greek, woman presented with a 1-year history of hair loss of the scalp. A complete dermatological and medical history was taken. The entire skin and mucous membranes were examined, and a complete general physical examination was performed. Routine laboratory tests included complete blood count, erythrocyte sedimentation rate, routine biochemistry, antinuclear antibodies and hepatitis-serology. Skin biopsy was taken from the affected area and the material was fixed in formalin and embedded in paraffin. Routine histologic sections were stained with Harris Hematoxylin-Eosin. Immunohistochemical examination was performed in 2 μm thick serial sections for the lymphocytic markers CD4, CD8, CD20, CD45RA, CD57 (DakoCytomation, DK). The staining procedure was performed on Bond Max™ autostainer (Visionbiosystems, Australia). The detection system used a polymer dextran molecule conjugated with horseradish peroxidase (Visionbiosystems). The direct immunofluorescence showed clumped deposition of IgM along the follicular basement membrane zone.

Key words: Frontal fibrosing alopecia, premenopausal, scarring alopecia, immunohistochemical analysis, immunofluorescence analysis

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1721-727 (2007)

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Eur. J. Inflamm. (DIF) in two steps method was used for the investigation of IgA, IgG, IgM, C3c (DakoCytomation) in the paraffin-embedded material. After incubation with primary antibodies, the immunocomplex was visualized using the sheep anti-mouse FITC-conjugated immunoglobulin (Novocastra). The immunofluorescence analysis was performed on a Zeiss microscope (Axioskop 2 plus HBO 100) equipped with a high quality objective and appropriate filter set (EX BP485/17 for FITC/spectrum green) and a computerized imaging system. Photographic images were captured with a computer-controlled digital camera and processed with a software system (FISH Imager™ METASYSTEMS).

RESULTS

History
A 36-year-old Caucasian, Greek, woman complained of hair loss of the scalp. The symptom started one year before presentation. She did not complain of itching, burning or exanthema in this area. The patient was otherwise healthy, without any symptoms of systemic disease, such as weight loss, arthralgia, or photosensitivity. Ten years ago, cholinergic urticaria had been diagnosed. Her daughter had keratosis rubra faciei.

Physical examination
On skin examination she demonstrated a smooth, shiny and atrophic band-like zone of 1 cm width on the frontal-temporal hairline. This zone contrasted the photoaged skin of the superior forehead, allowing speculation on the location of the original hairline. In this zone some small skin-colored hyperkeratotic papules without marked erythema and some intact hairs could be seen. The pull test was positive in the frontotemporal hairline and negative in other areas of the scalp. There were no clinical findings on the eyebrows or eyelashes. Further skin examination including nails, mucous membranes and the systematic examination of the other organs revealed no pathological findings.

Fig. 1. Dermatopathology. The epidermis was slightly atrophic with hyperkeratosis. In the upper dermis the hair follicles were destroyed and sebaceous glands were absent (1a). The remaining follicles showed a rimming of fibrous tissue (1b) and abundant lymphocytic infiltration around them (1c). Some lymphocytes invaded the follicular epithelium and are probably responsible for its destruction (1d). (Hematoxylin-Eosin stain; 1a magnification x 20, 1b magnification x 200, 1c magnification x 100, 1d magnification x 400).
Differential diagnosis.

The differential diagnoses were FFA and keratosis follicularis spinulosa decalvans.

Laboratory examination

Complete blood count, routine biochemistry, erythrocyte sedimentation rate, antinuclear antibodies and hepatitis-serology were without pathological findings.

Histologic examination and immunohistochemical assays

Dermal sections in Hematoxylin-Eosin stain showed slight epidermal atrophy and subepidermal
fibrosis. The hair follicles were destroyed and sebaceous glands were absent. The follicles were surrounded by fibrous bundles and lymphocytic infiltrations (Fig. 1a and 1b) which obscured the follicular epithelium (Fig. 1c and 1d). Immunohistochemical analysis revealed that the lymphocytic infiltration of the dermis was of T-cell origin, composed of CD4 and CD8 lymphocytes with a slight predominance of CD8 lymphocytes. The lymphocytic cell types which penetrated the follicular epithelium and seemed to be responsible for its destruction were almost exclusively CD8 lymphocytes. There were also CD4 helper lymphocytes in perifollicular infiltrations but very few invaded the hair follicle (Fig. 2a, 2b, 2c and 2d). Scattered lymphocytes of B-cell origin positive for CD45RA antigen were found inside and around the follicles (Fig. 2e and 2f). Lymphocytes positive for CD20 and CD57 were not observed.

Direct immunofluorescent examination showed clumped deposition of IgM along the follicular basement membrane zone (BMZ) (Fig. 3). IgA, IgG and C3c were negative.

**Diagnosis, treatment and clinical course**

Based on history, clinical, histological, immunohistochemical findings and laboratory examinations, the diagnosis of FFA was made. We treated the patient with clobetasol propionate lotion twice a day for 4 weeks. The hair loss was stopped and the pull test was negative. We continued this treatment once a day for 4 weeks and the patient remained in remission for two months. Unfortunately, a new flare of the disease started two months after the discontinuation of the steroid, and the disease was partially in control with tacrolimus ointment once a day. We advised our patient to have a regular control every 4 months.

**DISCUSSION**

Frontal fibrosing alopecia is a rarely occurring cicatricial alopecia and is considered a variant of lichen planopilaris. Classically, postmenopausal women are affected (1-3). There are only a few reports of the condition in premenopausal women (4-7) like our patient and only one in a man (8). The role of hormones in the pathogenesis remains obscure. Onset can occur after menopause, whether iatrogenic or occurring naturally, but the course of the disease remains unaltered by introduction of hormone replacement therapy. Serum androgen levels are normal (5, 7). FFA is usually insidious but can be self-limited or rapidly progressive (5). As differential diagnosis keratosis follicularis spinulosa decalvans, lichen planopilaris, Graham-Little syndrome, traction alopecia, ophiasis and androgenetic alopecia should be considered.

Results of a routine blood test in FFA are normal. Routine histopathologic, direct immunofluorescence and immunohistochemical evaluation of FFA are identical with lichen planopilaris. To date there is no evidence for an effective treatment of FFA. The disease is rare and double-blind placebo controlled studies are lacking. Topical, intralesional and oral steroids (2, 7), oral isotretinoin (5) and griseofulvin (5) showed some effect.

In our case, the characteristic distribution pointed to the clinical differential diagnosis, although our patient did not have the usually affected age. The clinical features along with the histopathologic findings established the diagnosis. The systematic clinical examination and the laboratory failed to reveal an underlying systemic disease. Stabilization of the disease was achieved with the use of a very potent topical steroid, although a new flare occurred two months after discontinuation of therapy, which is partially controlled with topical tacrolimus. In accordance with previous
studies by Kossard et al (5), immunohistochemical analysis revealed that the lymphocytic infiltration of the dermis was of T-cell origin, composed of CD4 and CD8 lymphocytes with a slight predominance of CD8. In our case, CD8 positive lymphocytes penetrated the follicular epithelium. This finding is particularly interesting, and further investigations are required in order to reveal possible pathogenetic mechanisms. DIF in lichen planopilaris is not uncommonly negative. Deposition of fibrinogen, IgM, as in this case, or less commonly IgA and C3, is seen along the follicular BMZ (9-10). The exact role of these antibodies is not yet clear.

In conclusion, the prompt recognition of FFA and the immediate therapeutic intervention, as with our patient, can interrupt the irreversible course of this cicatricial alopecia. Other forms of alopecias, and specifically cicatricial alopecias, must be considered in the differential diagnosis.

REFERENCES

SHORT REPORT

IL-13 NEUTRALIZATION MODULATES FUNCTION OF TYPE II POLARIZED MACROPHAGES IN VIVO IN A MURINE T-CELL LYMPHOMA

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IL-13 is a Th2 cytokine that suppresses the effector function and alters the phenotype and function of macrophages switching to alternatively activated or type II polarized macrophages. The type II polarized macrophages or M2 phenotype differ from normal macrophages greatly in terms of receptor expression, cytokine and NO production, that show tumor promoting function rather than tumoricidal function of classically activated macrophages. The chemokines CCL-22 and CCL-17 produced by either tumor cells or alternatively activated macrophages attract Th2 cells preferentially, which increase the local concentration of Th2 cytokines including IL-13 that further skewed the normal phenotype of macrophages at the site of the tumor micro-environment. Therefore, it is possible to restore the phenotype and function of alternatively activated macrophages by eliminating or blocking the activities of these cytokines. In the present investigation, we show that by blocking the activity/signaling of one of its major constituents IL-13, the iNOS expression and correspondingly NO production increases. The observation signifies its efficacy towards a novel approach for cancer therapy by modulating the function of tumor-associated macrophages (TAM) in vivo for the first time.

Interleukin (IL)-13 is an anti-inflammatory cytokine produced primarily by activated Th2 cells (1). It is also produced by human naïve and memory CD4+ or CD8+ T-cells (2), and exhibits pleiotropic effects on human peripheral blood monocytes and mouse granulocyte-macrophage colony stimulating factor (GM-CSF)-derived bone marrow macrophages (3). IL-13 has similar effects as IL-4, and both induce morphological and surface Ag changes in human monocytes and inhibit nitric oxide (NO) and pro-inflammatory cytokine production (4-7). It up regulates the expression of adhesion molecules and mannose-fucose receptor and the proliferation and migration of various immune cells (8-10). In addition, IL-13 induces the expression of various chemokines such as CCL22 (MDC; macrophage-derived chemokines) and CCL17 (TARC; thymus and activation regulated chemokines) that preferentially attract CC-chemokine receptor 4 (CCR4)+ T-helper cells, thereby amplifying polarized Th2 response in the tumor-bearing host (11-13). This infiltrating Th2 cell in tumor microenvironment increases local concentration of Th2 cytokines including IL-13. Furthermore, tumor cells secrete various soluble factors that effectively inhibit the tumoricidal function of macrophages and favor tumor growth and survival (14-16).

Macrophages are most abundant at the Th2 polarizing microenvironment of tumor site, where the function and phenotypes of macrophages change to polarized type II macrophages that differ greatly with classically activated macrophages.
or M1 phenotype, especially in terms of receptor expression, cytokine and chemokine production (17-18). IL-13 is a major constituent in the tumor microenvironment that significantly affects the function and phenotype of macrophages. It has been evidenced that IL-13 alternatively activates the macrophages and induces the arginase pathway for NO production with the generation of ornithine and polyamines that account for low NO production and reduced expression of inducible nitric oxide synthase (iNOS) (19). Therefore, it is plausible to assume that by eliminating or blocking immunosuppressive soluble factor(s), the function of alternatively activated macrophages can be restored and changed to the phenotype of classically activated macrophages. However, evidence in this regard is completely lacking.

It is evident that IL-13 has two receptor components; IL-13Rα1 and IL-13Rα2. IL-13 binds strongly to IL-13Rα1 when present in combination with IL-4Rα and induces IL-13 signaling in the cell (20-21). IL-13Rα2 attracts IL-13 avidly, but does not induce signaling, due to very short intracellular domain, and is designated as a decoy receptor (22). Utilizing the soluble form of IL-13Rα2 chain as an IL-13 inhibitor, the role of IL-13 in pathophysiology of asthma has been well characterized (23-24) as similar to the IL-13 blocking by IL-13 mAb (25). Therefore, in the present investigation, we sought to assess macrophage function in a T-cell lymphoma murine model, in terms of NO production and expression of iNOS, by blocking the activity/signaling of IL-13.

MATERIALS AND METHODS

Mice and tumor system

Inbred populations of BALB/c (H2b) strain of mice of either sex were used at 8 to 12 weeks of age. The mice were given food and water ad libitum under pathogen-free conditions and were treated with utmost human care in an approved animal room facility of the Department of Zoology, Banaras Hindu University. For tumor induction, the healthy mice were injected intraperitoneally with 1.5 x 10^7 DL-cells, a transplantable T-Cell lymphoma of spontaneous origin, in 0.5 ml PBS. The DL-cells for transplantation were obtained from ascitic fluid of DL-bearing mice by peritoneal lavage, where the yield of the cells is higher, and maintained in ascitic form in vivo by serial transplantation.

Reagents

Recombinant mouse IL-13 and monoclonal antibody of IL-13 were obtained from Invitrogen Inc., USA. The mouse recombinant IL-13Rα2 (539-1R) was purchased from R & D System Inc., Mn. The detection antibody conjugated with alkaline phosphatase was obtained from Bangalore Genie, India. Nitro-phenyl phosphate (NPP), Sodium azide (NaN₃), Diethanolamine and other chemicals were obtained from SRL, India.

Macrophage isolation and culture

Peritoneal macrophages were harvested from normal healthy, control DL-bearing and test group of DL-bearing mice of either sex by a standard method. Macrophages from both groups of mice at 12 days of DL-cell transplantation were harvested by peritoneal lavage using chilled serum-free culture medium RPMI 1640 after killing by pain-free cervical dislocation. Macrophage monolayer was prepared by incubating for 2 h in round plastic petri dishes (Tarson, India) at 37°C in CO₂ incubator. The culture was washed three times with lukewarm serum-free medium with gentle flushing to ensure that all DL-cells and other non-adhering cells were removed. More than 95% of the adhering population was that of macrophages, as determined by morphology and non-specific esterase staining.

The macrophages at 1.0 x 10^6 cells/well were seeded in 96-well flat bottom culture plates (Tarson, India) in RPMI 1640 culture medium supplemented with 10% heat inactivated FCS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were stimulated with 10 ng/ml of LPS for 24 h after pre-treatment in culture medium with or without 0.01 to 20.0 ng/ml IL-13 for 24 h at 37°C in CO₂ incubator, and further experimental analyses were carried out.

Systemic delivery of IL-13 Rα2

A group of 14 DL-bearing mice were injected intraperitoneally (i.p.) with 100 ng of IL-13 Rα2 dissolved in 0.5 ml of sterile PBS at 2nd day of post-DL-cell transplantation to neutralize systemic IL-13 level in DL-bearing mice. At 9th day of post-DL-cell transplantation, a second dose of IL-13 Rα2 in 0.5 sterile PBS was given by i.p. injection. After 12 days of IL-13 Rα2 i.p. injection in DL-bearing mice (test group), macrophages were harvested as described in the previous section. The mice injected only with sterile PBS at the corresponding day of IL-13Rα2 injection were treated as a control group.

Estimation of nitrite accumulation

Nitrite concentration in culture supernatants was determined by a standard micro plate assay. In brief, 50 µl samples were harvested from the conditioned medium
and treated with equal volumes of Griess reagent (1% sulphanilamide, 0.1% Naphthalene-ethylene-diamine dihydrochloride and 2.5% H$_3$PO$_4$) at room temperature for 10 min. The absorbance at 540 nm was monitored with a micro plate reader (UV/VIS spectrometer, Lambda2, Norwalk, Canada). Nitrite concentration was determined by using Sodium nitrite as a standard. The chemicals used for the preparation of Griess reagent were obtained from Sigma Chemical Co., St. Louis.

Western Blotting
For iNOS gene expression, macrophages ($10^5$) were lysed in ice-cold buffer containing 25 mM monosodium phosphate (pH 7.4), 75 mM NaCl, 5 mM EDTA, 1% Triton X-100, 100 µg/ml phenyl methyl sulfonyl fluoride (Merck), 10 µg/ml leupeptin, 10 µg/ml pepstatin, 20 µg/ml aprotinin and 10 µg/ml tyrosine (Sigma Co., St. Louis) inhibitor and centrifuged at 50,000 g for 20 min, at 4°C. The cytosolic proteins (6 µg/lane) were separated by 12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose filter paper and then immunoblotted with rabbit anti-iNOS monoclonal antibody followed by incubation with alkaline phosphatase conjugated antibody (Bangalore Genie, India) at a dilution of 1:5000. The colour was developed by using nitro blue tetrazolium reagent as described above.

Statistical Analysis
Each value represents the mean ± SE of three independent experiments in each group except for in vivo stimulation experiment where two independent experiments were conducted. Data were analyzed by using two-tailed student’s t-test on statistical software package Sigma Plot, version 5.0. Differences were considered statistically significant at $P < 0.05$.

RESULTS
IL-13 suppresses the production of NO in activated peritoneal macrophages
To assess the IL-13 inhibitory function on NO production of peritoneal macrophages (NMO), macrophages were pre-treated with IL-13 at different concentration in culture medium for 24 hours and stimulated with LPS (10 ng/ml), and it was found that IL-13 inhibits NO production in a dose-dependent manner, as has been reported previously (23). The LPS-activated macrophages without pre-treatment of IL-13 showed maximal accumulation of NO$_2^-$ in culture medium (26.7 ± 3.84 µM), and later with the increasing concentration of IL-13, corresponding suppression of NO$_2^-$ accumulation was observed (Fig. 1A). The most effective suppression was found with the dose of 10 ng/ml of IL-13. Consistent with previous observation, macrophages stimulated with LPS (10 ng/ml) in combination with IFN-γ (2 ng/ml) showed maximal production of NO and corresponding accumulation of NO$_2^-$ in the culture medium and with higher concentration of IFN-γ (more than 2 ng/ml); the suppressive effects were found to be reduced as per se (data not shown).

TAM from control mice (TAMc) show corresponding suppression of NO production
The macrophages from DL-bearing mice injected only with sterile PBS were activated with LPS without pretreatment of IL-13 and showed reduced NO production in comparison to macrophages harvested from normal healthy mice (Fig. 1A). The suppressive activity of IL-13 on TAMc seems to be more pronounced in comparison to NMO (normal macrophages) at the same condition which was found to be ~84% inhibition (Fig. 1B). This indicates that macrophages in the tumor-microenvironment has altered phenotype and has low responsiveness to LPS with respect to its normal counterpart. Furthermore, the TAMc were incubated with different concentration of IL-13 in the culture medium for 24 hours and thereafter stimulated with 10 ng/ml of LPS a dose dependent inhibition of NO$_2^-$ accumulation in the culture medium was found, a similar phenomenon as exhibited by normal macrophages (NMO). TAMc pre-incubated with medium alone and activated with LPS showed ~37% lower accumulation of NO$_2^-$ with respect to NMO at the same condition. In either case, non-stimulated and without IL-13 pretreated macrophages showed a basal level of NO$_2^-$ with almost no variation in the culture medium.

TAM from treated DL-bearing mice (TAMt) show enhanced production of NO
To assess the validation of assumption that the elimination or blocking of activity of Th2 cytokines may restore the function of macrophages, we checked it by injecting the DL-bearing mice with soluble non-signaling decoy receptor IL-13 Ra2 to block the activity/signaling of IL-13, as previously we have shown that the DL-bearing mice reflect a high serum as well as ascitic fluid IL-13 level (P. Deepak, et al., unpublished observation). Interestingly, we found that macrophages harvested from treated mice have
bands in the membrane. * indicates the significant lower density of iNOS protein in TAMc than the corresponding NMO and $ indicates the significantly higher density of iNOS protein in TAMt with respect to corresponding TAMc.

Fig. 1. Effect of IL-13 on nitrite production in TAMc. Macrophages isolated from normal healthy (NMO) and control group of DL-bearing mice (TAMc), at the cell density of 1.0 x 10⁵/well were stimulated with medium alone or medium containing LPS at 10 ng/ml for 24 hours after pre-treatment with medium alone or medium containing different concentrations of IL-13 for 24 hours. A. Nitrite production in culture medium was measured, and B. percent suppression of nitrite (NO₂⁻) accumulation in TAMc was determined. The values present mean ± SE of three independent experiments performed in triplicate and taken as significant at p<0.05. * indicates the significantly lower production of NO₂⁻ in corresponding TAMc than NMO in culture.

Fig. 2. Effect of IL-13 on nitrite production in TAMt. Macrophages isolated from control group of DL-bearing (TAMc) and test group of DL-bearing mice (TAMt), at the cell density of 1.0 x 10⁵/well were stimulated with medium alone or medium containing 10 ng/ml of LPS for 24 hours after pre-treatment with medium alone or medium containing different concentrations of IL-13 for 24 hours. A. Nitrite production in culture medium was measured, and B. percent induction of nitrite (NO₂⁻) accumulation in TAMt in corresponding condition were determined. The values present mean ± SE of three independent experiments performed in triplicate and taken as significant at p<0.05. * indicates the significantly higher production of NO₂⁻ in corresponding TAMt than TAMc in culture.
enhanced ability to produce NO on stimulation with LPS at 10 ng/ml (22.8 ± 2.65 µM) in comparison to TAM harvested from control mice (16.8 ± 2.66 µM). Pre-treatment with IL-13 at different concentrations for 24 hours showed a dose-dependent suppression of NO production (Fig. 2A) as in the previous case, and maximum suppression was found to be ~86.7% at 10 ng/ml of IL-13, more than the TAM harvested from control mice (PBS injected DL-bearing mice) indicating that macrophages are still sensitized to a polarized type II microenvironment. Here we found a significant increase in the release of NO$_2^-$ in culture medium, and it was found that LPS-stimulated TAM from treated mice (TAMt) without pre-treatment with IL-13 showed ~35.7% enhanced NO$_2^-$ accumulation with respect to TAM harvested from untreated or control mice (TAMc) (Fig. 2B). The increasing concentration of IL-13 showed increased suppression of NO production in NMO with respect to TAM harvested either from treated mice or control mice.

**IL-13 down-regulates iNOS protein synthesis**

Further to assess the effect of IL-13 on iNOS protein synthesis in all macrophage types; the macrophages from normal healthy mice, control DL-bearing mice and treated DL-bearing mice were incubated with medium alone or medium containing different concentrations of IL-13 (as indicated) for 24 hours and stimulated with 10ng/ml of LPS for another 24 hours, lysed, centrifuged at 4°C and culture supernatants were collected. The supernatants were resolved in 12% polyacrylamide in SDS-PAGE and immunoblotted with iNOS specific mAb. With each case, the iNOS expression
was found to be inhibited by IL-13. For the NMO (Fig. 3A), TAM harvested from control mice (Fig. 3B) and TAM harvested from treated mice (Fig. 3C) showed complete disappearance of iNOS protein in the blot. The iNOS protein was found to be expressed at 1.0 ng/ml of IL-13 in TAM from control and treated mice, whereas it appeared in the blot only after 0.1 ng/ml of IL-13 in culture medium of normal macrophages, showing the degree of inhibition of iNOS in phenotypically different macrophages.

The TAM from control mice incubated with medium alone and stimulated with LPS (10 ng/ml) showed ~59% appearance in respect to corresponding NMO (taken as 100% in the study). Induction due to IL-13 blocking in TAM harvested from treated mice (TAMt) showed ~85% appearance of iNOS protein in the blot (Fig. 4) in respect to NMO at same condition.

DISCUSSION

IL-13, a Th2 type cytokine, regulates the function of macrophages (7) and inhibits the expression of iNOS and subsequent production of NO (26). However, it has been found that IL-13 does not affect the mRNA expression of iNOS protein in peritoneal macrophages, but induces the expression of arginase-I that ultimately affect the L-arginine availability and subsequent inhibition of de novo synthesis and stability of iNOS protein (27). Furthermore, the replacement of L-arginine in the culture medium has been found to restore the iNOS protein level and NO production in IL-13 treated macrophages (28). The suppression of NO production and iNOS protein synthesis in IFN-γ/LPS stimulated macrophage cell line J774 (26) and other cell types (29-32) by IL-13 highlights the potential role in the alteration of macrophage function in Th2 dominating tumor-microenvironment. Our investigation validates the assumption that IL-13 Rα2 treatment induces the nitric oxide production and iNOS protein expression in TAM. Though little work has been done in this regard, the observation is in the line that polarized type II macrophages is not a type of macrophage but alternatively activated phagocyte cell population (33) and that its function can revert back in the Th1 polarizing cytokine milieu.

Our observation shows that TAM from control mice (PBS injected) respond more actively on pre-treatment of IL-13 than the normal peritoneal resident macrophages (NMO), and shows more
reduced production of NO and iNOS protein. The systemic delivery of soluble IL-13 Rα2 significantly enhances the production of NO and iNOS protein level in the macrophages. Similar to this observation, inhibitory function of IL-13 can be partially antagonized by increasing concentrations of IFN-γ as suggested by the much less impeded production of NO and expression of iNOS in IL-13 treated J774 macrophages stimulated with much higher concentration of IFN-γ (26). The observation is suggestive of the plasticity of macrophage function in Th1/Th2 microenvironment. On the above account, IL-13 seems to act as alternative macrophage activator, and IL-13 does not lead to alternatively activated macrophages as totally independent, stable and non-convertible functional states. Though, IL-13 blocking does not completely restore the function of TAM, it only partially, but to a greater extent, restores the function. The partial restoration of function may be due to the fact that in Th2-dominating tumor-microenvironment, several immunosuppressive factors such as IL-4, IL-10, TGF-β, PGE₂ etc. interact to the macrophages and alter the functional states of macrophages. The high inducing capability of IL-13 blocking by IL-13Rα2 indicates that their activities are interdependent. However, the mechanism of induction of iNOS expression and NO production in TAM obtained from IL-13Rα2 treated mice is not clear, and further investigation is required. It might be possible that the blocking of IL-13 activity/signaling inhibits the arginase-I expression (34) by shifting the balance of Th1/Th2 cytokines in the tumor-microenvironment (18, 28, 35-36) that leads to arginine consumption and reduction in NO production to IFN-γ (± LPS) (18, 28, 34), with (18) or without (28) alteration in the level of iNOS protein, as similar use of an arginase inhibitor during the pre-treatment period prevents the suppression of iNOS protein by IL-13 (27).

In summary, the IL-13 blocking in the tumor-bearing host, at least in part, restores the tumoricidal functional state of macrophages, and indicates the possibility of completely restoring the function of macrophages by blocking various immunosuppressive interdependent factors at the molecular level. Therefore, it is novel in the sense that it throws light on the possibility of novel anticancer therapeutic approach targeting most-abundant immune cell types at the tumor-microenvironment.

ACKNOWLEDGEMENTS

We are very thankful to the University Grants Commission, New Delhi, for the financial support to P.D. This work is a part of his PhD. We are grateful to Prof. S.M. Singh, School of Biotechnology, Banaras Hindu University, for his valuable suggestions and comments.

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CASE REPORT

ACUTE LEUKEMIA AND PREGNANCY

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Received July 4, 2006 – Accepted January 18, 2007

The combination of acute leukemia and pregnancy is infrequent. It is estimated to occur in less than 1 in 75,000 pregnancies. Maternal and fetal outcomes have improved substantially in recent years. In general, multi-agent chemotherapy is given as soon as the diagnosis of leukemia is established, even if it is in the first trimester. There are two important considerations in the management of a patient with leukemia during pregnancy, the mother who needs optimal cancer therapy and the developing fetus who could potentially be affected by the disease and/or the teratogenicity of antineoplactic agents. Vaginal delivery is preferable, and caesarian section is reserved for obstetrical indications only.

We present two cases of pregnant women who were diagnosed as having acute leukemia at 20 and 24 weeks of gestation. The first case suffered from acute lymphoblastic leukemia and the patient was started on multi-agent chemotherapy. A live male infant was delivered at 28 weeks and 4 days by caesarian section due to severe intrauterine growth restriction. The infant had apgar score 6/1 and 8/5 and made excellent progress in the following days. The mother recovered from the caesarian section without complications and is currently continuing her treatment.

The second patient was a 24 week pregnant woman who was diagnosed with acute myeloid leukemia. She was started on multi-agent chemotherapy and was delivered by caesarian section at 28 weeks. A live normal growth female infant was delivered with apgara scores 8/1 and 9/5. Both mother and infant made very good progress during the following days and the mother is currently continuing her treatment.

Both cases were diagnosed in our clinic within a period of six months. There are two important considerations in the management of a patient with leukemia during pregnancy, the mother who needs optimal cancer therapy and the developing fetus who could potentially be affected by the disease and/or the teratogenicity of antineoplactic agents. Overall, attention must be made to the drugs used and the stage of pregnancy. Delays or modifications in the therapy to ensure the birth of a healthy infant may affect the maternal prognosis adversely.

MATERIALS AND METHODS

First case report

A 23-year-old woman, primigravida, was transferred from a general district hospital to our clinic on the 02/04/2005. She was 20 weeks pregnant (with last menstrual period 17/11/2004) and had had an uneventful pregnancy up to this date. She had a past medical history of rheumatoid

Key words: acute leukemia, pregnancy, chemotherapy
arthritic and had undergone treatment with methotraxate when she was 17 years old. On admission, her symptoms were nose bleeding and left upper quadrant abdominal pain, which she had been suffering for three days. Her blood results revealed Ht: 23.2% Hb: 7.8% platelets: 17,000 and WBC: 14,000 (neutrophils 4%, lymphocytes 19% and blast cells 72%). On clinical examination and abdominal palpation she had hepatomegaly and splenomegaly. The ultrasound measurements agreed with gestation and the fetal heart was positive. A single live male fetus was found. The diagnosis of acute leukemia was made, after consultation with the hematologist department. An abdominal ultrasound revealed normal liver structures, hepatomegaly, splenomegaly, normal kidneys and bladder. From the immunphenotype of peripheral blood blasts and the myelogram, the diagnosis of acute B-LL was confirmed by the hematologists.

After liaison with the hematologists and discussion with the patient, we decided to continue with the pregnancy and to proceed with chemotherapy at the same time. In order to avoid any toxic effects to the fetus, the hematologists decided to use the following protocol of chemotherapy. The patient received four cycles of daunoblastine, oncovin and medrol tablets 16 mg three times daily, with one day administration of daunoblastine and the following day oncovin. She was also started on ciprofloxacin 500 mg and cetoconazole sir. The treatment lasted from 21/04/2005 to 10/05/2005. On 21/04/2005 we performed a detailed ultrasound which revealed no structural abnormalities, normal liquor volumes, normal growth at 21 weeks and 5 days and breech presentation. Initial investigations of the hematologists were satisfactory, but blood results and myelogram showed a relapse on 17/05/2005. In the meantime, due to abnormal glucose tolerance test, the patient was started on diet by the diabetologist.

Due to the relapse, and after discussion with the mother, the decision was made to then begin a new protocol with cyclophosphamide, vincristine, Adriablastine and decadron and to continue after delivery with subchorionic administration of methotraxate + cytocrine- arabinose+ solu-cortef. There was a good response to the treatment. On 05/05/2005 we performed an ultrasound. The findings showed a live infant of 24 weeks and 3 days, normal growth and liquor volumes and positive end diastolic flow (EDF). Two weeks later, due to poorly controlled diabetes, the patient was started on Actrapid+ protaphane by the diabetologist and the glucose levels were satisfactorily controlled. A gestational ultrasound on the same day showed normal growth for 27 weeks, estimated fetal weight 1,235 gr, cephalic presentation, liquor within normal limits but abnormal doppler measurements. There was negative E.D.F. and P.I: 1,716 for the umbilical artery, thoracic aortal P.I.: 2,705 and M.C.A.( mid-cerebella artery) P.I:1,705. There was obvious increased resistance in feto-placental and arteriovenous circulation. On 24/05/2005 the ultrasound findings were the same and we have decided to reassess her again in a few days. On 31/05/2005 she was 28 weeks pregnant and the A.F.I. (amniotic fluid index) was 8, umbilical P.I:1,588, thoracic aorta P.I: 1, 85 and M.C.A. P.I.: 1,075.

We continued observations by performing CTG’S three times daily. On 04/06/2005 she was 28 weeks and 4 days pregnant. Her CTG’S were abnormal with absent variability in three occasions. We decided to proceed to emergency caesarian section. A live male infant was delivered, with apgar score 6 in 1 minute, and 8 in 5 minutes, weighting 1,070 gr. The infant was transferred to the neonatal unit and had excellent recovery and progress during the following days. The placenta was preserved for stem cell recovery. The mother pre-operatively had Hb: 11, 8 PLT: 96,000 and WBC: 2,700 and was transfused in theatre with two units of red packed cells and 10 units of platelets. Her recovery from the operation was uneventful and she is currently continuing her treatment in the hematology unit. The results of her treatment are still to follow.

Second case report
The second patient was a 28-year-old primigravida who was attending our clinic. She had no past medical history. She was 24 weeks pregnant when a routine blood test revealed anemia, thrombopenia and leucopenia. The myelogram showed 80% blasts and the hematologists diagnosed acute myeloid leukemia. The ultrasound measurements agreed with the gestational age of 24 weeks and revealed no fetal pathology. After liaison with the hematologists and discussion with the patient, we decided to continue with the pregnancy and to proceed to chemotherapy at the same time. She was started on chemotherapy by the hematologists and received four cycles of Daunoblastine on days 1, 3 and 5 and of Cytarabine on days 1 to 5. The patient had a very good response to chemotherapy with no signs of infection during the phase of aplasia. The follow-up of pregnancy was made by performing weekly growth ultrasounds and by twice daily auscultation of fetal heart. At 28 weeks the ultrasound suggested normal fetal growth and breech presentation. In order to avoid any possible premature delivery during the phase of aplasia, we have decided, after liaison with the neonatologists and the hematologists, to proceed with delivery before starting the second phase of chemotherapy. We administered a total of 24 mg of betamethazone in two separate doses to the patient, and performed a caesarian section after 48 hours. A live female infant with no structural abnormalities was delivered. The
weight of the infant was normal for the gestational age, 1,235 gr, and the apgar score was 8/1 and 8/5. The infant was transferred to the neonatal unit and made very good progress. The mother recovered uneventfully from the caesarian section and was transferred to the hematology clinic were she is currently continuing her treatment.

DISCUSSION

The management of leukemia in patients who are pregnant and the effect of antineoplastic agents during conception and pregnancy have not been thoroughly investigated. The combination of acute leukemia and pregnancy is infrequent. It is estimated to occur in less than 1 in 75,000 pregnancies (1-2). Acute leukemia represents about 90% of leukemia co-existing with pregnancy. Acute myeloid leukemia accounts for about 60% and acute lymphoblastic leukemia for about 30% of cases. More than 75% of the cases are diagnosed after the first trimester (1-2).

In this report, we present two cases of leukemia and pregnancy which presented in our clinic with non-specific symptoms. The clinical manifestations of leukemia are very often non-specific, and most of these symptoms are common in normal pregnancies. With the progression of the disease, symptoms such as epistaxis, easy bruisability and recurrent infections should suggest a serious complication (3). The diagnosis is usually suspected from the peripheral blood smear and confirmed by bone marrow aspiration (4). There are two important considerations in the management of a patient with leukemia during pregnancy, the mother who needs optimal cancer therapy and the developing fetus, who could potentially be affected by the disease and/or the teratogenicity of antineoplastic agents.

In more recent times there is an improved survival rate with these malignancies, and in three-fourths of women who develop acute leukemia during pregnancy, remission can usually be induced with chemotherapy. Thus, maternal and fetal outcomes have improved substantially in recent years. There are numerous reports of successful pregnancies in patients aggressively treated with combination chemotherapy for acute leukemia (5-6). Acute leukemia and its therapy are associated with an increase in stillbirths, about 15%, prematurity 50%, and growth restriction (7). Despite being improved, perinatal outcomes are generally poor for leukemic women (Table I).

As for the management of these cases, because there is no evidence that pregnancy has a deleterious effect on leukemia, termination to improve the prognosis is not recommended, but it is a consideration in early pregnancy to avoid teratogenesis from chemotherapy. Most of the drugs used in the treatment cross the placenta and

<table>
<thead>
<tr>
<th>Reference</th>
<th>Number of pregnant women</th>
<th>Type of acute leukemia</th>
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<tr>
<td>Reynoso et al 6</td>
<td>58</td>
<td>AL</td>
<td>No mortality</td>
<td>50 LI (58% preterm)</td>
</tr>
<tr>
<td>Caliguri and mayer 1989 2</td>
<td>40, 20</td>
<td>AML, ALL</td>
<td>72% CR in AML, 76% CR in ALL</td>
<td>2 SA, 8 LI (8preterm), 13 TA</td>
</tr>
<tr>
<td>Ali et al 14</td>
<td>6, 4</td>
<td>AML, ALL</td>
<td>5 CR, 5 deaths during therapy</td>
<td>3 SA, 6 TA, 1 LI (SVD)</td>
</tr>
<tr>
<td>Chelghoum et al 9</td>
<td>31, 6</td>
<td>AML, ALL</td>
<td>34 CR (92%) during therapy</td>
<td>22 LI (13 SVD, 9CS), 2 SA, 13 TA</td>
</tr>
<tr>
<td>Current report</td>
<td>1, 1</td>
<td>AML, ALL</td>
<td>No mortality</td>
<td>2 LI (2 CS, 100% preterm)</td>
</tr>
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</table>
potentially could be teratogenic. Regarding the chemotherapy treatment we used in our cases, no serious long-term effects of in utero exposure in the second and third trimester were reported (8-9). Combination therapy engenders a greater risk than single agent therapy. Cytarabine and anthracyclines (except idarubicin) have not been associated with the occurrence of teratogenicity (9-10). In general, multi-agent chemotherapy is given as soon as the diagnosis of leukemia is established, even if it is in the first trimester (11-12). Overall, attention must be given to the drugs used and the stage of pregnancy. Delays or modifications in therapy to ensure the birth of a healthy infant may affect the maternal prognosis adversely (13). We believe that each patient should be examined individually, considering both the aggressiveness of leukemia and the stage of the pregnancy when therapy is applied.

Significant complications in pregnancy that include infection and hemorrhage should be foreseen at the time of delivery in women with active disease. Manifestations include anemia, neutropenia and thrombocytopenia (14). As for the mode of delivery, in both cases we performed cesarean section due to obstetric reasons. Generally speaking though, vaginal delivery is preferable, and cesarean section is reserved for obstetrical indications only (3, 15).

REFERENCES

CASE REPORT

OROFACIAL GRANULOMATOSIS: REPORT OF TWO CASES WITH GINGIVAL ONSET

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Received October 11, 2006 – Accepted January 19, 2007

Orofacial granulomatosis is a unifying term comprising a variety of clinical conditions involving the face and the oral cavity and histologically characterized by the presence of chronic granulomatous inflammation. Lip swelling and erythema are the most frequent clinical signs. We report on the clinical-pathological features and the management of two cases of orofacial granulomatosis characterized by gingival onset, without other local and systemic manifestations. The diagnosis of orofacial granulomatosis with gingival onset is made by the exclusion of other conditions exhibiting gingival inflammation and/or enlargement. Detailed medical history, haematological investigations and gingival biopsy are fundamental for the definitive diagnosis. Though infrequent, orofacial granulomatosis with gingival involvement should be considered in the differential diagnosis of hyperplastic gingivitis of uncertain origin.

Orofacial granulomatosis (OFG) may be defined as a group of conditions characterized by chronic, non-caseating granulomatous lesions involving the oral mucosa and the perioral tissues, which can also represent the oral manifestation of systemic granulomatous diseases (1-3). The clinical presentation of OFG is highly variable: facial swelling, lip swelling, angular cheilitis, vertical lip fissures, oral mucosal ulcerations, mucosal tags, cobblestoned oral mucosa, full-width gingivitis. Sometimes cervical lymph node enlargement can be observed (1-6).

OFG mainly occurs in children or adolescents, and can remain localized exclusively to the oral and maxillofacial region, at least for some time, but may be the first clinical sign of a systemic disorder or of advanced systemic disease (1, 3). Several conditions share with OFG similar clinical and histological features (e.g. Crohn’s disease, sarcoidosis, mycobacterial infection, foreign body reaction) and, therefore, the diagnosis might be challenging and only made by exclusion (7-8).

We report on two cases of OFG showing unusual primary gingival onset, which preceded other facial and mucosal signs, occurring in 2 white elderly women, and highlight the difficulties of differential...
diagnosis and therapeutic management.

MATERIALS AND METHODS

The patients were two white women (60 and 65 years old) presenting with chronic enlargement of the maxillary gingiva. A detailed medical history, along with extraoral and intraoral examination were carried out. Panoramic radiography, consecutive periapical radiography, hematological investigations (including erythrocyte sedimentation rate, serum folate, vitamin B₁₂, iron and transferrin, Ig A, IgG, IgM, IgE, serum angiotensin converting enzyme, C₁INH, chest radiography, Mantoux test, Kveim test, radioallergosorbent test (rast) and patch test were performed, as well as gingival biopsy. The gingival biopsies were performed using a biopsy punch of 3.5 mm of diameter and the surgical specimens were fixed in 10% neutral-buffered formalin for 48 hours and embedded in paraffin. Sections from the paraffin blocks were cut and stained with hematoxylin and eosin (H&E), followed by observation under polarized light, and Ziehl-Neelsen.

RESULTS

Both patients were affected by localized hyperplastic gingivitis of the maxillary attached gingiva (Fig. 1). In case 1 it was associated with folding of the buccal mucosa and lingua plicata (fissured tongue). In case 2 only lingua plicata was associated with gingival enlargement at first clinical presentation, but upper unilateral lip erythema and enlargement developed within 2 months, as did bilateral cervical swelling, confirmed by ecography as being due to lymph node enlargement. All clinical-serological investigations showed no relevant abnormalities with the exception of moderately increased neutrophil count, increased erythrocyte sedimentation rate and rheumatic factor.

In both patients histological examination on H&E stained sections revealed non-caseating granulomatous inflammation (Fig. 2, 3), collections of histiocytes in micro-granulomatous structures (Fig. 4) and multinucleated giant cell (Fig. 5) within the superficial lamina propria of the gingiva, intermixed with an intense lymphocytic infiltrate. Ziehl-Neelsen-stained sections were negative for mycobacteria and no bi-rifringent foreign body materials were detected by polarized light microscopy. The histological features were indicative of granulomatous gingivitis with presence of multinucleated giant cells.

In view of the results of the clinical, hematological and histological investigations, a final diagnosis of OFG presenting as hyperplastic gingivitis was rendered in both cases. Topical therapy was instituted with 0.05% clobetasol ointment in a 1:1 ratio with orabase, applied twice daily for three weeks using soft plastic gingival trays, and with 2% miconazole gel, applied three times daily for four weeks. In both patients the gingival lesions completely resolved within 4 weeks (Fig. 6).

DISCUSSION

The clinical manifestations of orofacial granulomatosis (OFG) can be various. Bilateral or unilateral progressive lip enlargement is usually the first and most frequent clinical sign, followed by other manifestations (4, 9-10). However, gingival involvement only very infrequently precedes other orofacial manifestations. It usually presents as a full-width gingivitis or as a localized gingivitis, in the absence of plaque that is the usual cause of such a reaction.

The diagnosis of granulomatous gingivitis can be challenging for the clinician, the pathologist and the patient. The differential diagnosis includes: Melkersson-Rosenthal syndrome (9-11), Crohn’s disease (12), tuberculosis or leprosy, sarcoidosis (7-8), Wegener’s granulomatosis (13), acquired and hereditary C₁INH-related angioedema (14), hypersensitivity reactions (15-16), deep fungal infections, and foreign body reactions.

Complete medical history and physical examination are mandatory to exclude the presence of concomitant systemic involvement by other granulomatous lesions. In this view, red blood cell examination, white cell count and differential, erythrocyte sedimentation rate, levels of serum angiotensin converting enzyme, electrolytes, folate, vitamin B₁₂, albumin, iron, transferrin, immunoglobulins, chest radiography, and Mantoux test were included. Gallium scans, bowel biopsy and radiography, RAST and patch tests, may be helpful to rule out concomitant extra-oral or systemic granulomatous inflammation. The final diagnosis usually is achieved following gingival biopsy that
shows prominent lymphocyte accumulations in the superficial lamina propria, associated with microgranulomas mainly composed by histiocytes and occasional multinucleated giant cells. The latter show multiple nuclei that usually are dispersed at the periphery of the cytoplasm. Similar morphological features may be encountered in tuberculosis, sarcoidosis, Crohn’s disease and foreign body reaction that can be excluded, based on the lack of acid-alcohol resistant mycobacteria and birefringent foreign particles under polarized light, and in view of the lack of extra-oral (lung, bone, salivary glands and intestine) involvement by the granulomatous inflammation.

Fig. 1. Full-width hyperplasia of the upper anterior gingiva, involving the attached gingiva and the interdental spaces.

Fig. 2. Panoramic view of gingival granulomatosis: well-formed, non-caseating granulomas within the superficial lamina propria, diffuse lymphocytic infiltration with edema and prominent dilated blood vessels are detectable. (Hematoxylin and eosin, original magnification x50).
Fig. 3. At higher magnification, sub-epithelial lymphocytic infiltration, epithelioid histiocytes and multi-nucleated cells within a non-caseating granuloma are evident. (Hematoxylin and eosin, original magnification x100).

Fig. 4. Non-caseating histiocytic granulomas of different size are evident in the sub-epithelial lamina propria of a patient with orofacial granulomatosis. These may be composed by very few cells or comprise a collection of several histiocytes. In this specific area multinucleated giant cells are not detectable. (Hematoxylin and eosin, original magnification x200).
Adequate diagnosis of OFG is mandatory to reach satisfactory therapeutic results: there is, in fact, a minimal chance that OFG may spontaneously regress (2) and prolonged therapies are often needed. These are based on multi-drug combinations, including corticosteroids and anti-mycotics, even in the absence of evident fungal infections, both because mycosis may not be clinically, culturally or histologically manifest and because they may possibly develop following prolonged corticosteroid therapy. It should be stressed, however, that cases of OFG resistant to prolonged treatments do exist (4, 8) and, in such cases, complete clinico-radiological re-evaluation may be necessary to further exclude gingival involvement by systemic diseases.

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