THE DELAYED HYPERSENSITIVE RESPONSE TO *CANDIDA* ANTIGEN AS ASSESSED BY THE VIRAL PLAQUE ASSAY AND SKIN RESPONSE IN PATIENTS WITH CHRONIC VAGINITIS

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ABSTRACT

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A method by which specifically sensitized thymus derived (T lymphocytes) lymphocytes involved in delayed type hypersensitivity can be quantitated *in vitro* has been described (1, 2). The method makes use of the fact that when lymphocytes are exposed *in vitro* to an antigen to which they had been previously sensitized *in vivo*, they undergo a transformation which then makes them capable of supporting the replication of virus. By enumeration of plaque areas on a viral sensitive monolayer of target cells where discrete viral infected T lymphocytes landed, specific antigen reactive cells in a population can be quantitated.

Using this method and skin reaction the delayed hypersensitive response to *Candida* antigen in patients with chronic *Candida* vaginitis and a control group was investigated. The patients with vaginitis were divided into two groups, those with and without symptoms. All patients with symptomatic

infections demonstrated positive skin tests and activation of lymphocytes *in vitro* when exposed to *Candida* antigen. These results suggest a possible allergic mechanism contributing to the symptomatic infection. Asymptomatic patients all had no skin response and no activation of lymphocytes *in vitro*. Control donors skin response correlated with activation *in vitro*.

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TABLE OF CONTENTS

LIST OF TABLES

ARTICLE

LIST OF FIGURES

FIGURE Page

ARTICLE

INTRODUCTION

The delayed hypersensitivity response in patients with chronic vaginitis was investigated. These patients were diagnosed by a gynecologist's office as having chronic vaginitis caused by Candida, a yeast-like fungus commonly found as a normal saprophyte on the human body. The treatment of vaginitis caused by Candida has been a problem to gynecologists. Effective antifungal agents are available; however, a certain number of these women are apparently incurable. That is, treatment leads only to temporary relief of symptoms and temporary absence of the organism. A large percentage of these patients can be found to have hormonal imbalances, reinfection from other sources, or have a disease that is known to cause defects in cellular immunity to infection.

The delayed hypersensitive response to this organism is quite common, occurring in approximately 50-80% of the population. This indicates that Candida is a highly potent allergen, easily inducing delayed response as a result of its saprophytic nature or by subclinical infection. Several minor irritations have been associated with an allergic response to the presence of Candida in the area.

The human body's defense against fungal disease is known to be mainly cellular. In the presence of an effective specific cellular immunity to Candida, an infection by Candida should be limited. A small study was therefore conducted on a group of patients with various types of Candida vaginitis and a control group. Their cell-mediated response to Candida antigen, in vivo and in vitro was investigated. In vivo, skin testing was done with response noted at 48 hours. In vitro, a quantitative method was used, an adaptation of the viral plaque assay (1,2).

Correlation between the in vitro assay for delayed hypersensitivity and the skin testing was found. Patients with symptomatic vaginitis all had positive delayed reactions in vivo and in vitro, suggesting a possible allergic type response to the presence of the organism in the vagina. In contrast, patients with an asymptomatic type infection with Candida displayed a non-reactivity to the antigen, suggesting a possible tolerance due to the presence of the antigen over an extended period of time. Control patients demonstrated correlation with in vitro and in vivo testing.

LITERATURE REVIEW

The Genus Candida

"At least seven species of Candida have been found associated with pathogenic manifestations in man" (3). In order of pathogenicity they are C. albicans, C. tropicalis, C. stellatoidea, C. pseudotropicalis, C. parapsilosis, C. guelliermondii, and C. krusei.

The most commonly found and the most pathogenic is C. albicans (synonyms, Oidium albicans, Monilia albicans, and Endomyces albicans). Specialized chlamydospore agar will produce structures which can be used for the identification of C. albicans. These chlamydospores are large (7-17 microns), thick-walled round cells appearing at the tips and sides of the mycelia. Incubation of yeast phase cells in serum at 37° for 1-2 hours will produce a budding elongation in C. albicans. These germ tubes are also used for the identification of this organism. Sugar fermentations and assimilations are used to separate the members of this genus.

In culture at room temperature or at 37° on Sabouraud glucose agar or corn meal agar, colonies of C. albicans are white, creamy and opaque. These colonies are usually completely in yeast phase, thin-walled budding cells, 2-6 microns in

diameter. Pseudomycelium, elongated yeast cells, as well as true mycelium are produced occasionally under proper conditions.

Pathogenicity

Candida is often present as a saprophyte in or on normal healthy persons (see Table I); however, it may become invasive and cause disease due to some predisposing factor. It has been called a disease of the diseased (3) (see Table II). Invasion may occur superficially in the mouth, vagina, intestines, lungs, and skin and nail folds. It also may cause a systemic infection, invading the central nervous system, circulatory system, respiratory system, digestive tract or the urinary system. As a systemic infection, it is very often fatal (5).

Most recently immunosuppressive therapy, corticosteroid therapy and antibiotic treatments have introduced an alarming amount of fatal or debilitating fungal infections (6,7,8).

Skin and mucous membrane infections are much more common than systemic infections. Infection of vaginal and oral mucosa is characterized by multiple white to cream-colored patches with some inflammation. The patches consist of a dense growth of yeast and hyphae forming a pseudomembrane. Skin lesions are erythemic and moist, occurring most commonly in skin folds and clefts. Nail infection is a painful, inflammatory condition in which an exudate may form. Eyes, ears, lungs and systemic infections are less common.

Table I. Incidence of *Candida albicans* in man (3,4).

Newborn infants--mouth swabs	1- 4%
Oral cavity	30-50%
Vagina--pregnant	20-40%
non-pregnant	4-16%
Feces	10-30%
Normal skin	1%

Table II. Predisposing factors to infection by *Candida* (3).

A. Hormonal disturbances and other idiopathic states	
Diabetes	Agranulocytosis
Leukemia	Bronchiectasis
Hypoparathyroidism	Malformation of the urinary tract
Pernicious anemia	
Hypoadrenocorticism	Ulceration of the digestive tract
Aplastic anemia	
Carcinoma	Debility
Moribund state	Malabsorption
	Malnutrition
B. Pre-eminently receptive states	
Pregnancy	Maceration of skin
Infancy and old age	Skin surface contact with carbohydrates
Carbohydrate-rich diet	
C. Drug therapy	
Antibiotics	Contraceptive drugs (See Ref. 17)
Corticosteroids	
D. Infectious disease	
Tuberculosis	Typhoid and other enteric infections
Chronic bronchitis	
Influenza	Bacterial endocarditis
E. Surgery	
Open heart operations	Tooth extractions
Bowel resections	Eye operations (corneal grafts)
Colostomy	Ear operations (skin grafts)
F. Accidental introduction of *Candida* by intravenous injections or indwelling urinary catheters	
Blood transfusions	
Glucose saline drips and other supportive fluids	
Drugs, especially in addiction	
G. Accidental trauma	
Eye injury	
Burns	

It has been claimed that the two growth phases of C. albicans, yeast phase and mycelial phase represent the change of the organism from a saprophytic to an invasive or parasitic state on a host organism (9). Host tissue factors or constitutional factors may also account for the presence of the different forms and thus the presence of mycelial forms may not definitely indicate Candida infection.

Endotoxin associated with the yeast cell has been described as contributing to its pathogenicity (10). The release of an endotoxin by dying cells may cause local irritation and damage, thus allowing further penetration of the organism. A capsule associated with the mycelial phase has been proposed as a location for the endotoxin, which may explain the incidence of mycelia in infected areas. Various preparation of Candida cell extracts have been demonstrated to contain a substance which will produce erythema and dermatitis in guinea pigs, rabbits, and humans (11). Large doses of extract were also able to cause death in experimental animals suggesting that endotoxin may be a possible mechanism for shock seen in Candida septicemia (7).

Electron microscopic studies investigating the ultrastructural relationship of Candida albicans in oral candidiasis, revealed the presence of yeast cells outside, penetrating, and inside the epithelial cells (12).

Candida vaginitis

Pruritus, white or yellow epithelioid or curdy secretions, occasionally thrush patches, erythema of the vulval vestibule, and pH usually acid from 3.8 to 5.0, all describe the characteristic vaginal infection produced by Candida albicans (13). Normally the infection is superficial, associated with the vaginal secretions and little inflammation (14). The normally saprophytic organism is quite commonly found in the vagina without causing the above clinical picture; however, under certain conditions it may become invasive and cause vaginitis. Increase in glycogen content as in pregnancy and diabetes, antibiotic and other drug therapy, allergy, immunological deficiency, irritation as well as those conditions listed in Table II have been associated with vaginal candidiasis; however, definite causative relationship is often so vague as to elicit a suggestion that it is emotional (15). The incidence of vaginitis in relation to use of oral contraceptives has been proven insignificant (16,17).

These infections usually respond to topical application of anti-fungal agents. Adequate and properly used treatment is apparently the key to a cure in most uncomplicated cases. Quite often after treatment there is a reoccurrence of infection. This can be attributed to any number of factors mentioned above as well as reinfection by the sexual partner or fecal contamination, insufficient use of medication, or possibly deepseated invasion.

Candida Infection in Mice

Artificially induced infections in animals help to better understand the pathogenesis of disease by establishing model infections. In mice a model Candida infection can be induced by intraperitoneal and intravenous injections of C. albicans. This infection is very often fatal if the organism injected is in high enough concentrations or if a mouse-virulent strain is used. The major site of involvement is the kidney. This predilection for the kidney is accounted for by its slower clearance of the organism from the body. The organism penetrates the renal tubules and proliferates. This intratubule position may protect it from normal cellular defenses and allow it to gain a fatal foothold. Other organs, lung, spleen, heart, and liver, have been found able to mount an effective cellular response and clear the organism (18).

Active and passive immunization, tolerance induction, artificial diabetes, thymectomy, and a variety of chemicals have been investigated for their effect in relation to severity or rate of mortality in Candida infection in mice and other animals. Active and passive immunization gave a significant degree of protection as indicated by increased survival rates. Cortisone, artificial diabetes, anti-lymphocytic serum, oxytetracycline, metabolic imbalance, and thymectomy all caused an enhancement of infection. Tolerance was induced cellularly and did not effect the antibody response (19,20, 21,22,23,24,25,26,27).

An artificial Candida thigh lesion in mice was also described. This would seem to better imitate cutaneous infections in man. The induced infection was self-limiting and localized. Various chemicals, such as antibiotics and cortisone, were tested for their effects on the lesion (28,29).

The ultimate insult to the mouse population was, however, an experimental vaginal infection induced in mice (30).

Humoral Immunity

Problems in establishing the significance of the isolation of Candida species in an infection and in the diagnosis of a possible underlying Candida systemic infection have led to many studies on antibody titers in candidiasis in an effort to correlate positive serologic findings with overt infection.

Precipitating, agglutinating, and complement-fixing antibodies to Candida have been found in humans (31). Precipitins appear to have little correlation with infection. In one study precipitating antibodies were found in 48% of healthy volunteers and in 69% of patients with mucocutaneous candidiasis (32). Indirect fluorescent antibody titers showed somewhat higher titers in candidiasis as compared with healthy subjects (33). Comaisch suggested that high agglutination titers indicated the presence of Candida somewhere on the body as a result of his studies; however, some overt infections had low titers (34). A recent study (35) attempted to derive some correlation from antibody measurement and infection by using

three methods, precipitation, agglutination, and fluorescent antibody titer. They recommend that titers should be run on all hospital patients that have an increased chance of acquiring candidiasis, "high risk" patients, and watch for rising titers. The measurement of increasing titer appears to be all that is worthwhile in antibody determination as the presence of antibody is not necessarily indicative of disease.

Specific local antibody systems, secretory IgA, have been suggested as a possible defense mechanism on mucosal surfaces. The inducement of local IgA by C. albicans in vaginal secretions (36) and measurement of specific antibody in saliva (37) and in vaginal secretions (38) have been demonstrated, but their protective influence not definitely established.

Apparently immunity is primarily of the cellular type rather than humoral. Patients with Swiss-type agammaglobulinemia, a thymic abnormality, have a high incidence of mucocutaneous candidiasis. In contrast, there is a low incidence of Candida infection in Bruton form of agammaglobulinemia, where cellular immunity is intact (39). Classified immune deficiencies such as these and other thymic or cellular abnormalities associated with Candida infection have established the immune defense against this organism at the cellular rather than humoral level.

Non-Immunologic Humoral Mechanisms

Iron-unsaturated protein found in serum (transferrin) and in exudates and transudates (lactoferrin) have been found to compete for available iron with pathogenic organisms, C. albicans included, inhibiting their growth. Roth and Goldstein originally reported this as C. albicans growth depressing substance found in normal subject's serum. Its action did not appear to be immunologic and was diminished in patients with acute blood dyscrasias (40). Kirkpatrick et al. reported on iron-unsaturated lactoferrin located in milk, tears, and intestinal fluid and found that it impaired the replication of C. albicans. This inhibitory effect was lost when saturated with iron. He suggested that this may be a mechanism for mucosal protection; however, the absence of lactoferrin in several patients with mucosal candidiasis was not found (41).

A second humoral factor called clumping factor has been reported. This factor, present in almost all normal children and adults under fifty years old, induces filament and germ tube formation in C. albicans and C. stellatoidea and eventually causes clumping if the organism is in high enough concentration. Children demonstrated that this factor does not kill the organism and that it is inhibited by Candida specific IgG. It was also concluded from his studies that specific antibody against Candida does not affect the organisms growth (42).

Further studies show that this factor is unaffected by heating at 60°C for 1 hour, by dialyzing for 24 hours, antibiotic treatment, or by glucose; however, trypsin abolished its activity. Gamma globulin alone did not induce clumping and normal levels of clumping factor were found in hypogammaglobulinemia (43).

A clumping inhibitory or interfering factor was also reported in this same study. In patients who were apparently lacking clumping factor, 85% demonstrated an inhibition of clumping when mixed in 1:2 dilution with normal sera containing clumping activity. It was suggested that lack of clumping factor and/or presence of interfering activity may be a more sensitive indicator of Candida infection than agglutination titers. Patients with candidiasis in this study showed 72% with elevated agglutinin titers but 95% showed reduced clumping activity or interfering factor.

Candida allergy

An allergic mechanism for some active forms of Candida infection has been suggested (44). The very fact that a large percentage of the population show a positive delayed skin response, as high as 83% in 50 or older subjects, indicates that it is an organism that easily induces a hypersensitivity in an exposed individual. Exposure apparently occurs very often due to the saprophytic nature of the organism or a subclinical infection. It is suggested that some transient or

minor irritations such as mucous colitis, chronic urticaria (45), pruritus ani, and some asthematic conditions (46) may be the result of an allergic response to the presence of the Candida antigen (44). Patients with "irritable colons" do show increased delayed skin responses to Candida.

In contrast however, patients with denture stomatitis caused by Candida had fewer immediate and delayed skin responses than did the control group (47). The elicitation of skin response in Candida infection has shown this trend in other studies also (48). Since delayed skin tests are a reflection of cellular immunity, the negative skin tests in these infected patients could represent a defect that allowed the Candida invasion.

It is apparent that there may be an allergic or hypersensitive mechanism operating in some cases of contact with Candida. To what extent or in which cases this occurs can perhaps only be evaluated by immediate and delayed skin testing. As a diagnostic tool, however, in determining the presence of Candida infection, skin testing is unreliable.

Cellular Immunity

Since it has been shown that humoral immunity, antibodies, have little or no effect as a defense mechanism against Candida infection, the underlying defense must be cellular. Cellular reactivity to antigen involves three main steps:

(1) antigen processing and recognition, (2) production of humoral mediators by specifically activated lymphocytes, (3) activation of macrophages and changes in vascular endothelium. The activated lymphocytes have been demonstrated to be thymus derived lymphocytes or T cells. These become activated by specific processed antigen and undergo changes to release a variety of factors which are able to mediate cellular immunity. These mediators (49) have been demonstrated to affect macrophages, neutrophils, and eosinophils. These in turn when activated are responsible for phagocytosis and destruction of intracellular pathogens. Factors are also released by these activated T cells that cause cytotoxicity and vascular permeability, as well as other activities.

Defense against most fungal and viral and some bacterial infections are mediated in this way. A defect or decreased effectiveness in any step of the cellular response could allow invasion of these organisms to cause disease. This has been conclusively shown in many cases where the immune response is depressed or defective. Viral and fungal infections are very common and in very severe deficiencies are often the reason for fatality.

Abnormal phagocytic activity is found with diabetes, leukemia, and corticosteroid therapy, all of which are associated with decreased resistance to infection (7). Some investigation of phagocytic mechanisms in Candida infection have been

reported (50,51). Depressed phagocytosis or ineffective intracellular digestion in neutrophils have been found in myeloperoxidase deficient cells and in chronic granulomatous disease where there is a deficiency in oxidative response to phagocytosis. Both of these disease states have a high incidence of Candida infections.

In contrast to lymphocyte activation of phagocytes, it was reported recently that eosinophils were stimulated to phagocytize by antigen-antibody complexes in Candida infection. They found phagocytic activity to parallel antibody production and demonstrated the presence of antigen-antibody complexes inside the eosinophils (52).

Cutaneous Delayed Hypersensitivity

The delayed type skin response is characterized by an erythemic induration occurring from 48-72 hours after intracutaneous injection of a specific extract to which the person has been previously sensitized. Histologically, a dense collection of polymorphonuclear cells may be present as well as increased vascular permeability and other acute inflammatory reactive processes.

Negative response to antigen in an individual indicates one of three things: (1) insufficient exposure to induce hypersensitivity, (2) excess of pathogen in tissue so that anergy develops, (3) inability of host to respond to specific antigen. This inability to respond has been demonstrated to

occur at several levels. Some patients have been found to exhibit negative skin tests but have lymphocytes capable of response to specific antigen or mitogens in in vitro testing.

Chronic Mucocutaneous Candidiasis

A recent NIH conference called chronic mucocutaneous candidiasis "model building in cellular immunity" (53). The disease is characterized by chronic often widespread infection of skin, nails, and mucous membranes. On the basis of skin lesions, the infection is classified as Candida granuloma, chronic localized candidiasis without granuloma, or chronic diffuse candidiasis (39). The various immunological defects that have been found associated with this disease entity run the gauntlet of possible defects in the cascade of events in the cellular immune response. Many patients have been investigated who have a history of chronic candidiasis without systemic involvement, underlying endocrinopathies or classifiable immune deficiencies. It appears that their immune system is intact enough to prevent most infections from overwhelming them; however, slight defects in cellular immunity allow our primary opportunistic fungal infection, Candida, to invade mucosal and skin surfaces. These are very often long-standing, repeated infections, only temporarily relieved by treatment.

No singular defect has been distinguished in these patients; however, most demonstrate generalized cutaneous anergy. Studies of their defects help to identify factors that

help to establish commensal relationships between a host and potential pathogen, such as Candida. Migration inhibition factor (MIF) deficiency has been associated in several cases, possibly because this is easily assayed; however, other undetected defects could be present (54,55,56,57). It was also suggested, that an inhibiting serum factor for MIF may be present causing this deficiency as found by Canales et al. (58). They investigated a serum factor, present in a chronic candidiasis patient, that blocked normal proliferative response of the small lymphocyte in a normal subject. An unidentified defect in a patient was noted in 1968 by Marmor and Barnett, apparently a factor deficiency or defect in macrophage activation (59). The main observation in this case was cutaneous anergy with normal lymphocyte stimulation by non-specific mitogen. Desensitization due to chronic exposure to Candida antigen was suggested in another study (60). An antibovida antibody was found in another patients serum. It was found to cross react with lymphocytes inducing decreased lymphocyte function, thymic dysplasia, and possibly related IgA deficiency (61).

These defects, associated with chronic candidiasis, all reveal a few of the defense mechanisms necessary to maintain the saprophytic nature of Candida. Further studies on reconstitution of the immune systems of patients like these are quite interesting, but not within the scope of this paper (53,56).

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ARTICLE

The Delayed Hypersensitive Response to *Candida* Antigen as Assessed by the Viral Plaque Assay and Skin Response in Patients with Chronic Vaginitis

By

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SUMMARY

The delayed hypersensitive response to *Candida albicans* in patients with chronic *Candida* vaginitis and in a control group was investigated. *In vivo*, skin reactivity was assessed. *In vitro* the viral plaque assay was adapted to assay quantitatively the lymphocyte response to *Candida* antigen. Correlation was found between the *in vitro* assay and the skin response. In addition patients with symptomatic vaginitis all had positive delayed reactions to *Candida* antigen, suggesting a possible allergic mechanism. Patients with asymptomatic infection showed no activation or response to *Candida* antigen. Control donors responded *in vitro* according to the response of the skin test.

INTRODUCTION

Several experimental systems for detecting delayed hypersensitive responsiveness *in vitro* have been reported (Ruddle, 1972). These assays detect humoral mediators released by sensitized lymphocytes during a cellular immune response or measure the incorporation of radioactive thymidine. The cell which these systems detect is the T cell (thymus derived lymphocyte) which has been found to mediate delayed hypersensitivity or cellular immunity.

A system was needed in which the actual number of these T cells in a sample could be enumerated. An assay was reported in which individual specifically sensitized T lymphocytes could be quantitated by their ability to replicate virus when they became activated by specific antigen (Bloom and Jimenez, 1970; Jimenez *et al.*, 1971b). Normal resting lymphocytes were reported in earlier studies to be incapable of replicating virus; however, exposure to a nonspecific mitogenic agent, such as phytohemagglutinin resulted in lymphoid cells capable of replicating virus (Edelman and Wheelock, 1966; Willems, Melnick, and Rawls, 1969). Utilizing this concept, Bloom's method quantitates the T lymphocytes specific for a particular antigen in a sensitized animal or human. The antigen to which

the animal has been sensitized is incubated with the lymphocytes *in vitro*. During incubation the antigen-specific T cells are transformed to an activated stage which supports viral replication. Transformation involves increased synthesis of cell RNA, morphological alterations, and biochemical changes (Willems, Melnick, and Rawls, 1969). The activated cells are exposed to virus for a period long enough to allow viral penetration, excess virus is removed and the cells are plated on a lawn of virus sensitive target cells. The enumeration of clear plaque areas formed by viral lysis on the indicator lawn of monolayered cells at the site where a single viral infected T cell had landed provided a quantitative estimate of specifically sensitized T cells in the original population.

The cellular immune status of *Candida* antigen in a select group of individuals was assessed using this viral plaque assay along with skin testing for delayed response. The patients selected for this study presented a picture of chronic candidal vaginitis. Some were found clinically to support large and constant populations of *Candida* in their vagina with little or no symptoms. Others were found with symptomatic type infections, pruritus, curdy secretions, and erythema. Both groups were classified as chronic in that proper treatment resulted in only a temporary disappearance of the organism.

A large percentage of patients like these may be found to have hormonal imbalances, reinfection from other sources, or have a disease or being treated with some agent that is known

to cause defects in cellular immunity to infection. Because of the therapeutic problems associated with management of these patients, an investigation of the immunological status of a representative group was designed to provide insight into the problem. Little documented correlation exists between the status of Candida infection and detection of antibodies (Seelig, 1966). Some non-immunologic humoral and local factors against Candida have been described, but their importance remains to be established (Louria et al., 1972; Kirkpatrick et al., 1971). Therefore, only the cellular immune status of these patients was investigated in vitro and in vivo.

MATERIALS AND METHODS

Human lymphocyte donors

Several patients with chronic symptomatic or asymptomatic vaginal candidiasis were selected by a Lansing, Michigan gynecologist. Control donors were selected from volunteer graduate students and staff at Michigan State University.

Skin testing

Skin tests were performed with Candida allergenic extract, 500 protein nitrogen units (PNU) per ml. (Hollister-Stier Laboratories), injecting 0.01 ml. intradermally and reading for induration at 48 hours.

Lymphocyte separation

Thirty ml. of heparinized peripheral blood (Heparin: 33USP units/ml. blood, benzyl alcohol preservative, Upjohn Company) were allowed to settle 1½ to 2 hours until approximately 35-40% of the plasma had separated. The plasma was expressed from the syringe into a sterile glass wool (Scientific Products) column and allowed to incubate on the column for 15 minutes. The column was then drained and washed with PBS (phosphate buffered saline) containing 5% calf serum (Grand Island Biological Company).

Lymphocyte culture

After centrifugation and removal of the supernatant fluid, the cells were counted and plated with CMRL medium (Grand Island Biological Company), 2×10^6 cells/ml. in plastic tissue plates (Falcon Plastics No. 3001 or No. 3002). The CMRL medium contained 20% fetal calf serum, 100 units/ml. penicillin, 100µg/ml. streptomycin, 2mM L-glutamine, 20µg/ml. L-asparagine (Grand Island Biological Company) and 5×10^{-6} M. 2-mercaptoethanol (Click, Benck, and Alter, 1972). Cultures were prepared with or without Candida antigen (Hollister-Stier, 60,000 PNU/cc.) at concentrations reported to produce MIF, 100, 50, 10 PNU/2×10^6 cells (Goldberg et al., 1971). Incubation of these cultures was for a period of four days at 37° in 10% CO_2.

Target cell cultures

Baby hamster kidney (BHK) cell layers were prepared by dispensing 5 x 10^5 cells/5ml. in MEM (Hank's base, Grand Island Biological Company) with 10% calf serum into 60 mm. plastic tissue culture dishes. A complete monolayer had formed at the time of assay.

Viral incubation and removal

Cultured lymphocytes were washed once and incubated with New Castle Disease virus (NDV) at a multiplicity of infection of 25 for 2 hours at 37°. The cells were then washed three times and incubated with rabbit anti-NDV for 1 hour at 4°C.

Plating of viral-infected cells

Various dilutions of cells, 10^2-10^4, were plated, 0.2 ml./plate, on the BHK monolayers. Agar overlay, incubation, and staining were done according to the method of Bloom (Bloom and Jimenez, 1970).

Calculations

The difference in PFU/10^6 (plaque forming units) cells with and without antigen was calculated by subtracting the PFU of the control from the PFU of the cells with antigen. The percent activation was determined by dividing the difference in PFU by the control PFU times 100%. Calculation of the change in PFU (Δ PFU) between cells with antigen and control without antigen, in part negates high backgrounds as they

are relatively constant in a particular assay system. For sake of comparison of results, PFU was converted to a percentage figure which more accurately indicates the activation of a particular group of cells in comparison to the control.

Figure 1 illustrates the experimental design used for the viral plaque assay.

RESULTS

Twenty-one *in vitro* experiments with *Candida* vaginitis in ten patients along with four normal control individuals were assayed for cellular response to *Candida* antigen. These results are presented in Table I. Skin responses were found to be consistent within the two groups of vaginitis patients. Symptomatic vaginitis patients, group A, all had positive skin tests as assessed by the presence of induration at 48 hours. Diameter of induration ranged from 3 to 6 mm. Asymptomatic patients, group B, all had negative skin response to *Candida* antigen. *In vitro*, PFU generated from lymphocytes incubated with or without antigen show high backgrounds and wide variability in the patients tested. Comparison of the percent of activation to the control values of cultures without antigen for each patient, demonstrates activation in the presence of a positive skin response in group A. The geometric mean of activation in group A was 47%. Group B demonstrates a negative activation with negative skin response. The geometric mean of

Figure 1. Viral Plaque Assay--Experimental Design for Enumeration of *Candida*--specific Human Thymus Derived Lymphocytes

A. Lymphocytes from heparinized peripheral blood, purified to approximately 80% concentration on a glass wool column.

B. I. Incubated with *Candida* antigen.
II. Control--no antigen.
Incubated for 4 days at 37° in 10% CO_2. Antigen activates specifically sensitized lymphocytes.

C. Incubation of lymphocytes with New Castle Disease Virus (NDV) for 2 hours at a multiplicity of infection of 25. Virus is absorbed into activated lymphocutes.

D. Several washings with large volumes of PBS with 5% calf serum and neutralization of excess virus with rabbit anti-NDV.

E. 10^2-10^4 cells plated on monolayers of Baby Hamster Kidney (BHK) cells and overlaid with agar.

F. Cells stained with a vital stain and areas of viral target cell lysis, where individual infected lymphocytes landed, were enumerated.

G. Background plaques subtracted from plaques counted with antigen activated cells. Percent of activation as compared to control was calculated.

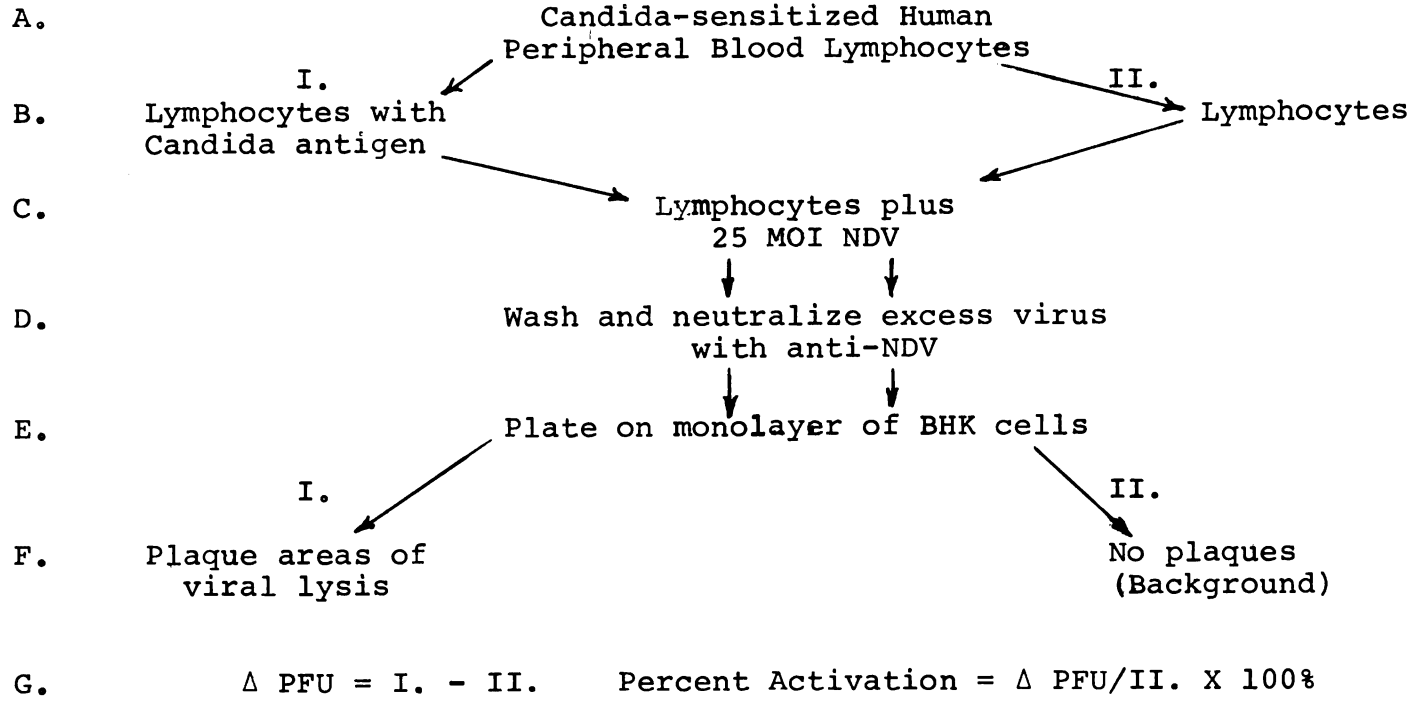
A.
Candida-sensitized Human
Peripheral Blood Lymphocytes
I.
II.
B.
Lymphocytes with
Candida antigen
Lymphocytes
C.
Lymphocytes plus
25 MOI NDV
D.
Wash and neutralize excess virus
with anti-NDV
E.
Plate on monolayer of BHK cells
I.
II.
F.
Plaque areas of
viral lysis
No plaques
(Background)
G.
Δ PFU = I. - II.
Percent Activation = Δ PFU/II. X 100%

Figure 1.

activation in this group was -33%. Control donors, groups C and D, none of whom presented a history of *Candida* infection, responded *in vitro* with activation or non-activation correlating with a positive or negative skin response. Skin test positive controls showed a geometric mean of activation of 47%. Controls with negative skin response demonstrated an activation of -4%.

Statistical correlation was found to exist between skin response and percent of activation of the lymphoid cell population *in vitro* (see Table II). Correlation between groups A and C and A and D are marginally significant; however, correlation at the 97% confidence level is found between all positive skin responses and activation of lymphocytes by antigen *in vitro* and all negative skin responses with no activation *in vitro*.

A listing of the percent activation of each patient tested is given in Table III. With several patients *in vitro* testing was repeated once after approximately a four-month interval. These are subclassified as *a* and *b*. The results of repeated testing demonstrate a fluctuation in immunologic status during a period of time or deviation in test results from assay to assay. Patients in group A with symptomatic candidiasis demonstrated a range of activation from 5% to 666%. Group B, asymptomatic patients, showed activation in a range of -54% to -1%. Group C included two control donors

Table I. Results of Viral Plaque Assay with *Candida* antigen Geometric Mean and Standard Error and Skin Test Response

Group	Experimental Status	N	*Candida* Skin Test	PFU$_6$ x 10^3 /10^6 cells with ag.	PFU$_6$ x 10^3 /10^6 cells control	Δ PFU x 10^3	Percent Activation
A	Symptomatic *Candida* vaginitis	8	+	205 ± 216	105 ± 188	59 ± 55	47 ± 79
B	Asymptomatic *Candida* vaginitis	4	-	529 ± 532	785 ± 508	-196 ± 137	-33 ± 12
C	Control	4	+	290 ± 206	135 ± 113	65 ± 154	47 ± 117
D	Control	5	-	62 ± 127	64 ± 130	9 ± 6	-4 ± 12

Table II. Statistical Correlation by Student T Test Between Test Groups

A vs B	P < 0.10	d.f. 10
A vs D	P < 0.10	d.f. 11
A + C vs B + D	P < 0.025	d.f. 19

A. Symptomatic Chronic Vaginitis: + skin test

B. Asymptomatic Chronic Vaginitis: - skin test

C. Control: + skin test

D. Control: - skin test

Table III. Percent Activation Results of Individual Subjects' Lymphocyte Cultures with *Candida* Antigen

Group A		Group B		Group C		Group D	
Number	Percent Activation	Number	Percent Activation	Number	Percent Activation	Number	Percent Activation
1	64	6	-21	10a	3	12	10
2	250	7	-54	10b	17	13	15
3a*	31	8	-43	11a	506	14a	-48
3b*	13	9	-1	11b	188	14b	5
4a	48					14c	21
4b	666						
5a	5						
5b	109						

*a and b are samples from the same subject taken at approximately a four month interval.

who had positive skin responses. Donor number 11 showed very high activation although she had no history of active Candida infection. Three negative skin test donors in group D exhibited activation in a range of -48% to 21%. Donor number 14 was repeated three times with an increasing activation from -48% to 21%.

DISCUSSION

The cell detected in the viral plaque assay is a non-dividing cell that allows viral replication only when acticated by a specific antigen to which it had been previously sensitized (Bloom and Jimenez, 1970). This cell is thought to be at the effector end of the cellular immune response in that it does not undergo further blastogenesis in the presence of antigen; however, some change does occur in the cell or on the surface which then allows viral replication. This T lymphocyte is involved in the delayed response *in vivo* as the response *in vitro* correlates with the *in vivo* response as assessed by the skin test.

Other studies to detect cell mediated reactions *in vitro* have found correlation with skin response. Studies with macrophage inhibitory factor (MIF), cytotoxicity, and thymidine uptake have shown this correlation (Salvin, Nishio and Gribik, 1970; Ruddle and Waksman, 1968; Folb and Trounce, 1970). In addition soluble mediators produced by T cells in

culture have been able to cause delayed type skin reaction *in vivo* (Jimenez and Bloom, 1971a).

In the experiments just reviewed, high, variable backgrounds were noted. These high backgrounds were relatively constant in a particular assay and thus by subtracting the control populations PFU from the antigen stimulated population the percentage of activated cells in the population may still be quantitated. High backgrounds may be attributed to the following: The patient may have had activated cells to other antigens present in the peripheral blood when drawn for study. This would be expected in a highly allergic individual. In addition it is known that some other cell types, such as granulocytes, and monocytes, will allow replication of virus. Samples used in this study contained approximately 20% contaminating white cells and may have allowed non-specific viral replication. Also, varying amounts of red cell contamination may have carried virus over to the viral-sensitive monolayers. Mechanics of the procedure also may have been at fault. Ineffective washing or antisera neutralization may have allowed excess virus to remain in the sample.

The results indicate a correlation between the *in vitro* assay and the delayed skin reaction. Patients with symptomatic vaginitis all had positive delayed reactions *in vivo* and *in vitro*, suggesting a possible allergic type response to the presence of *Candida* in the vagina. The possibility exists with these patients that allergic desensitization may help

alleviate some of the irritation associated with this condition. Some work has been reported on desensitization treatment of patients with chronic candidal vaginitis. These authors report success in relieving symptomatic infections by desensitization (Kudelko, 1971; Hosen, 1972). Patients with asymptomatic infection with Candida all exhibited a non-reactivity to the antigen, suggesting a possible antigenic suppression induced by the presence of antigen over an extended period of time.

Skin testing for delayed type hypersensitivity still appears to be the simplest method of assessing cellular immunity to a particular antigen. The viral plaque assay and other in vitro methods are applicable when using antigens that can not be used in vivo, tissue antigens, tumor antigens, drugs and to assess a negative skin test. As a routine clinical test the viral plaque assay is too complex in its performance. Its usefulness lies in its ability to quantitate lymphocyte response to a particular antigen.

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CONCLUDING REMARKS

These studies of patients with chronic vaginitis have shown some mechanisms involved in the pathogenesis of this infection which could be helpful in the treatment and management of these patients. Little has been said in the article to be submitted for publication regarding patients introduction to the project, interviewing, and culturing which was done before actual immunological testing was started. Permission to proceed with this investigation was obtained from the Michigan State University committee on use of human subjects for research. Patients were selected by Dr. G. Daugharty, a local gynecologist, for this study on the basis of their having a candidal vaginitis which had not responded to repeated treatment. Introductory letters were sent to patients explaining all that they would undergo in the course of the project (see copy Appendix A). On the first appointment consent forms (Appendix B) and interview forms (Appendix C) were completed and cultures were taken as follows:

1. Vaginal: A moistened speculum was inserted. A sterile swab was used to wipe the posterior vagina and around the cervix. A second swab was innoculated in the same way and used for a wet preparation for trichomonas, gram stain and pH.

A vaginal washing was also taken. Ten milliliters of sterile water was inserted with a glass pipette and bulb and aspirated out. For culture this was concentrated by centrifugation and the sediment innoculated.

2. Rectal: A moistened sterile swab was inserted into the rectal orifice.

3. Mouth: The patient swirled ten ml. of sterile water in her mouth which was then collected in a sterile tube. This was concentrated by centrifugation before innoculation for culture.

4. Skin lesions: Any recurrent skin lesions were scraped or the exudate collected.

Cultures were examined primarily for the presence of Candida albicans; however, an overwhelming preponderance of a specific organism was noted.

Second appointments were introduced to each patient with a letter (Appendix D). Skin testing and thirty ml. of blood were drawn at this time. Instructions on reading the skin test were given to each patient and the results obtained by phone.

The results compiled from the interview form revealed no consistent pattern or predisposing factor in the patients history that was common to all investigated. It was of interest, however, that of the patients with symptomatic infections approximately 60% had histories of allergy. This observation helps substantiate a possible allergic mechanism operating in these cases.

Culture results were far from conclusive, probably as a result of long-standing treatment or deep-seated invasion of the organism. Patients were asked to refrain from medication or douching for a week prior to the first appointment. Several of the patients felt they had an infection at the time of culture, although culture results very often did not verify this. This again suggests an allergic phenomenon where the degree of hypersensitivity developed in some of these patients may have allowed small numbers of the organisms to cause irritation and symptomatic infection.

In conclusion, it is felt that this is a sadly neglected area in the field of gynecology. Patients with chronic Candida vaginitis are not uncommon, and perhaps too often their problem is dismissed as mental after repeated unsuccessful attempts at therapy. Allergic desensitization with Candida antigen should be attempted in patients with history of allergies and with positive delayed skin response to Candida antigen. This therapy may prove to be the answer to the irritation associated with the presence of the saprophyte Candida albicans in the vagina.

APPENDICES

APPENDIX A

PATIENTS INTRODUCTORY LETTER

Dear

We are conducting a study concerning the immunology and other related factors in chronic Candida vaginitis (yeast infection). The research is to be carried out by Mrs. Betty Tavella, a medical technologist currently doing graduate work at Michigan State University, under the supervision and assistance of G. Daugharty, M.D., an obstetrician and gynecologist; R. Ghannam, M.D., an allergist; and several doctors from the Department of Microbiology and Public Health at M.S.U.

Volunteers are needed to participate in the study. A control group consisting of women having no symptoms of vaginitis and a group of women having chronic vaginitis will be asked to complete a questionnaire. This questionnaire is to elicit history and symptoms. The data will be compiled to obtain statistical information regarding vaginitis and associated problems. This history will also help us to decide whether you fit into the limited scope of our project.

Several studies will be done including vaginal, rectal, and mouth culturing; blood testing and skin testing. At your first appointment the following will be done: (1) The vaginal culture will be obtained by swabbing the upper vagina. Also a sterile washing will be taken to retrieve the vaginal secretions. This will be done by rinsing the vagina with a sterile solution. (2) Rectal culture will be obtained by inserting a moistened swab about an inch in the rectum. (3) Mouth culture will be procured by asking you to swirl a sterile solution around in your mouth and collecting in a sterile tube.

Skin testing and blood drawing will be done at a second appointment if the results of the first indicate that your problem is in the area we are researching. You will be notified of our decision concerning this. Skin testing is done by pricking the forearm with a needle and applying or injecting just beneath the skin a selected material in solution. If you have had or have an infection or been immunized against the material you will get a positive reaction. This reaction consists of a small red swelling which should disappear within a week. This should not be too uncomfortable and is the best test available to determine the state of your immunological response. At this time also approximately 20 cc of blood will

be drawn with a needle from a vein in the inner side of the elbow region.

Women willing to participate in the testing can be assured that all information will be kept confidential, and that she may at any time during the study withdraw from further participation. Participation as seen at present should involve only these two appointments at your convenience.

Vaginitis, which is an infection of the vagina, has had little research in the area of the immunological effects and consequently little is known about a condition which affects many women at some time in their life. The study which we propose, hopefully may provide some additional clue as to why some women are affected repeatedly and others never have the problem. We are not promising a cure as a result of our study and certainly can not guarantee that the women participating in the study will be relieved of their chronic vaginitis through participation; however, whatever can be learned from the study may aid in the better treatment and understanding of the problem.

The following consent form is required by law to be signed by persons cooperating as a volunteer in an investigative project. We will be glad to answer any questions you may have concerning this study.

Sincerely,

(Mrs.) Betty Tavella M.T. (ASCP)

APPENDIX B

PATIENT CONSENT FORM

I hereby agree of my own free will to participate in a study conducted by G. Daugharty, M.D., R. Ghannam, M.D. and the Department of Microbiology and Public Health, M.S.U., concerning the immunology and other related factors in chronic Candida vaginitis.

I understand that interviewing, culturing, blood sampling and skin testing may be done in the study and that I have the right to withdraw at any time from further participation.

I further understand that I am not being guaranteed a cure, only that I will have contributed to the better understanding of chronic vaginitis.

_____

Date _____

Number _____

APPENDIX C

QUESTIONNAIRE TO COMPILE INFORMATION REGARDING CHRONIC CANDIDA VAGINITIS

Number_____ Birthdate_____ Height_____ Weight_____

The following information is confidential. This data will be compiled to obtain statistical information regarding vaginitis and associated problems. Please circle the most correct answer. The interviewer may discuss certain answers with you for further clarification.

General Health

1. How do you rate your general health? Good Average Poor

2. How do you rate the general health of your teeth and oral cavity? Good Average Poor

3. Do you or any member of your immediate family have any of the following:

Please check if yes	You	Family
Diabetes		
Thyroid problems		
Anemia		
Leukemia		
Cancer		
Arthritis		
Unusual susceptibility to infection		

4. Have you ever had surgery? Yes No

5. Do you take any medications regularly (prescribed or otherwise)?

	Yes	No
If Yes:	1	2 3
What kind?		
For what purpose?		
How long?		

6. Any medications taken within the last month, other than those described above? (antibiotics, skin ointment, etc.) Yes No

7. Do you have any type of persistant skin problems--skin eruptions, boils, mouth sores, especially skin eruptions on the hands and feet? Yes No

Number_____

8. Have you had any laboratory tests done within the past year? Yes No

9. Any history of allergic disease in your or your immediate family? [hay fever; allergy to foods or drugs; contact allergies, poison ivy, metals (watch bands, rings)] Yes No

10. Record of immunizations: (please check if you have had)

Diptheria	[ ]	TB skin test	[ ]
Tetanus	[ ]	Mumps	[ ]
Pertussis	[ ]	Measles	[ ]
Smallpox	[ ]	Typhoid	[ ]

Other _____

Gynecological

1. Number of pregnancies _____ (include miscarriages, abortions)

2. Are you pregnant now? Yes No

3. When did you complete your last pregnancy? _____

4. Do you experience any difficulty or pain when having intercourse? Yes No

5. Please list contraceptive measures used:

	1	2	3
Type:			
Length of time used:			

6. Present contraception used: _____
   If oral contraception what brand name? _____

7. Do you use vaginal douches regularly? (other than medically prescribed) Yes No

8. Do you use feminine hygiene sprays? Yes No

9. When using toilet itssue, do you wipe from the rectal area into the vaginal area? Yes No

10. Do you wear cotton underpants? Yes No

11. Menstrual history:
    When started (age)_____
    Is flow heavy? Yes No
    Breasts painful? Yes No
    Severe cramps? Yes No
    Length of period_____ Time from end of period to beginning of next period_____

Number_____

12. Have you ever had a vaginal infection? Yes No
    (If several, please describe first observed)
    a. How long ago did this occur? _____
    b. Symptoms, please circle yes or no if present:

Burning	Yes	No
Itching	Yes	No
Swelling	Yes	No
Discharge	Yes	No
Bleeding	Yes	No
Frequent urination	Yes	No
Urinary tract infection	Yes	No

    c. Factors that may have influenced or caused the infection:
    (pregnancy, illness, environment, irritation, intercourse)
    List:

    d. Relation of infection to menstrual flow: Please circle

    Immediately following Immediately preceding
    During menstrual flow Midway between menstrual flows

    e. Treatment used: _____

    If a prescribed medication was used, was it used adequately and for the prescribed length of time? Yes No

    f. Diagnosis of infection (if known): _____

    How was diagnosis made? (culture, smear) _____

13. Have you ever had a urinary tract infection Yes No
    If yes, was it associated with a vaginal infection? Yes No

14. Any reoccurrence of vaginal infection? Yes No
    If yes, please answer the following:

    a. How often do you get a reoccurrence? _____

    b. What is usual pattern of infection? (concurrent urinary tract infections, symptoms, etc.)

    c. Diagnosis of infections, if known, and how they were made:

    d..Usual treatment, or what different types have been used:

Number_____

14. Have you ever had any other genital infections, other than those described above? (tubes, ovarys, venereal diseases)

Yes No

15. Has your male partner(s) ever had any type of penis discharge, inflammation, or sores.

Yes No

16. Do you feel that you have a vaginal infection at this time?

Yes No

Symptoms:

Have you used any treatment? Yes No

Any comments?

Have you read our introductory letter and signed the consent form?

Yes No

THANK YOU FOR YOUR COOPERATION.

APPENDIX D

SECOND APPOINTMENT INTRODUCTION LETTER

October 11, 1972

Re: Research project on the immunology of chronic Candida vaginitis.

Dear _____:

I am now starting to schedule previously seen patients to have skin testing done with Candida antigen. These appointments will be in the office of Dr. Rasem Ghannam, 919 Chester Street, across the street from Dr. Daugharty's office. Dr. Ghannam is an allergist and will supervise the skin testing to be done at this time. This appointment will take approximately one hour, during which time the skin test will be done and thirty milliliters of blood will be drawn. In 48 hours after the initial skin test a second reading must be taken. To alleviate the necessity of a second appointment, you will be instructed how to read the test and convey the information to me by telephone.

The blood test that I will be doing is a new quantitative method to determine your degree of sensitivity to Candida antigen. This will then be compared with normal individuals to possibly give some insight into the immunologic problem involved. Since it takes a week to run one test, I will only be scheduling 2 to 4 patients a week; therefore, the appointments will be continued over the next few months and there may be some delay in my contacting you.

This will complete my part in the study on Candida infections. I will attempt to draw up a short summary of my results for those who participated and mail it to you at a later date.

Please advise me if you are taking any type of cortisone drug, as this will interfere with the skin testing. Also, please do not consume any coffee or drug that contains caffeine for 24 hours before the appointment as this interferes with the blood test. It is not necessary to discontinue taking any other medication before the appointment.

I will be contacting you by telephone to schedule these appointments.

Sincerely,

Phone: 332-2349

(Mrs.) Betty Tavella M.T. (ASCP)