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UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION
Vienna International Centre, P.O. Box 300, 1400 Vienna, Austria
Tel: (+43-1) 26026-0 · www.unido.org · unido@unido.org

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OF BIOLOGICALS IN LATIN AMERICA

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LATIN AMERICA REGION

Technical report: Immunodiagnostics for Infectious Diseases: The Case of Latin America*

Prepared for the Latin America Region
by the United Nations Industrial Development Organization

Based on the work of Paul F. Basch
Department of Health Research and Policy
Stanford University

Backstopping officer: M. Polievktov, Chemical Industries Branch

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* This document has not been edited.

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INTRODUCTION

Resources for health are severely limited everywhere, but in the developing countries of Latin America, where external debts are placing additional constraints on social services, the need for optimal efficiency is particularly acute. Despite limitations of financial and human resources and the frequent unavailability of sophisticated laboratories, diagnostic tests can contribute to better health through more appropriate prevention and treatment for individuals and more effective disease control in communities.

This report will be limited to a discussion of immunodiagnostic procedures and reagents with special reference to the countries of Latin America; however, the technical methodologies are universal and the social and economic implications have broad application in other developing countries of the world. Although immunodiagnostic procedures have wide application in diagnosis of malignancies, autoimmune diseases, and other chronic conditions, this report will concentrate on the immunodiagnosis of infectious agents. Table 1 lists some of the more significant pathogenic organisms in Latin America for which immunodiagnostic procedures are, or could be, useful.

Although numerous diagnostic procedures are currently available, there is a great need to improve and extend them. For example, hepatitis nonA-nonB (HNANB)is very common in developing countries. The enterically transmitted pathogen has about a 20% case fatality rate in pregnant women. Parenterally transmitted, HNANB may account for more than 70% of transfusion-transmitted hepatitis. However, no adequate diagnostic tests have yet been developed (WHO, no date).

CHARACTERISTICS OF DIAGNOSTIC PROCEDURES

Diagnosis is a process of ascertainment leading to a decision about the status of health or illness in an individual, of disease in a population, or of a pathogen or contaminant in a specimen. Diagnostic methodologies form the basis for the definition and clarification of almost all health-related issues. Besides their patient-related and screening functions, these procedures represent the critical validating element in trials for safety and efficacy of vaccines and drugs, in epidemiologic surveillance, and in many decisions about the necessity, feasibility, and value of public health activities. The major purposes and uses of diagnostic procedures are given on Table 2.

The usual result of a diagnostic procedure is the classification of an individual or a specimen into a particular category having health significance. These categories must be precisely pre-defined in technical, and often cultural and legal terms, because the consequences of placement in one or another category may be great. For example, on an individual level, a diagnosis may lead to a therapeutic program that may be lifesaving, but could also be lengthy, costly, and dangerous. The increase in tissue and organ transplantation, including blood transfusion in the age of AIDS, has reinforced the need for accurate diagnostic procedures.

Basic biomedical research may be lead to unforeseen diagnostic applications. For example, monoclonal antibody and DNA technology have given rise to ingenious and powerful diagnostic tools for clinical use, but also in food hygiene and in personal identification for legal purposes, that were unanticipated by their originators.

Table 1

SOME INFECTIOUS DISEASES OF IMPORTANCE IN LATIN AMERICA

Viral Diseases
- Arboviruses, including Dengue and Yellow fever
- Aseptic meningitis
- Cytomegalovirus
- Hepatitis A, B, nonA,nonB
- Herpes viruses
- Human Immunodeficiency virus
- Influenzas
- Measles
- Mumps
- Poliomyelitis
- Rabies
- Respiratory syncytial virus
- Rubella
- Viral encephalitides
- Viral gastroenteritis, including Rotaviruses

Bacterial Diseases

Enteric
- Campylobacter
- E. coli infection, various
- Salmonella
- Shigella

Systemic
- Meningococcal meningitis
- Plague
- Pneumococcus
- Tetanus
- Tuberculosis
- Typhoid

Sexually transmitted
- Gonorrhea
- Syphilis

Rickettsial Diseases
- Chlamydial diseases
- Lyme disease
- Q fever

Fungal Diseases
- Aspergillosis
- Blastomycosis
- Coccidiodomycosis
- Cryptococcosis

Protozoan Diseases
- Amebiasis
- Chagas' Disease
- Giardiasis
- Leishmaniasis
- Malaria
- Toxoplasmosis

Helminthic Diseases
- Ascariasis
- Filariasis including Onchocerciasis
- Hookworm infection
- Schistosomiasis
- Strongyloidiasis
- Trichuriasis

TABLE 2

SOME PURPOSES OF DIAGNOSTIC PROCEDURES

1. As applied to Individuals:
   - presence and extent of illness or disability
   - suitability for work, school, military service, etc.
   - eligibility for insurance policy, pension, etc.
   - probability of commitment or incarceration
   - lifetime experience with an infectious agent
   - current state of infection, whether symptomatic or not
   - the precise identification of an infectious agent
   - state of susceptibility, resistance or immunity to an agent
   - definition of a pathologic process
   - prognosis; likelihood of future clinical disease
   - likelihood of adverse effect of a medical procedure
   - effectiveness of a prior immunization or treatment
   - degree of infectiousness and risk to others
   - need for immunization, chemotherapy, surgery, or other procedure
   - likelihood of having, or transmitting, certain genetic characters
   - determination of paternity
   - identification for legal purposes

2. For screening of biologicals and products:
   - protection of transfusion- related blood supply
   - protection of donor sperm for insemination
   - ascertainment of characteristics of organs and tissues for transplantation
   - detection of contaminants in vaccines or other biologicals
   - safety of meat, eggs, dairy products and other foods

3. Epidemiologic and Community Applications:
   - distribution of a disease or agent within the population
   - types and roles of reservoir hosts and vectors, if any
   - economic impact of an agent, disease or condition
   - need for a control program in the community
   - determination of efficacy of a vaccine or drug
   - importation and transport of animals and plants

Source: Adapted in part from Basch, 1989

General principles of diagnostic tests

Several kinds of immunodiagnostic procedures are described in the following sections. These have certain characteristics in common with all other diagnostic tests. Figure 1 illustrates the four possible outcomes of a diagnostic test to ascertain the status of an individual or a specimen: two outcomes are correct (true positive and true negative), and two are incorrect (false positive and false negative).

The sensitivity of the test is the proportion of true positives among all those in which the condition is really present. The fewer false negatives, the more sensitive is the test; if there are none at all, its sensitivity is 100%.

However, the test may also incorrectly determine some people or specimens to be positive. The proportion of such false positives defines the specificity of the test, which reaches 100% if the test does not misclassify any true negatives as positives.

The sensitivity and specificity of diagnostic procedures are always estimates, because the real truth is never known with certainty. Newly devised tests are validated primarily by comparison with previously available diagnostic procedures.

The actual predictive value of a test depends not only upon its inherent sensitivity and specificity but also upon the prevalence of the condition in the population. The probablility that the condition really exists in a person (or specimen) with a positive test is sometimes called the positive predictive value; and conversely the probability that a person classified as negative really does not have the condition may be called the negative predictive value.

FIGURE 1

OUTCOMES AND CONSEQUENCES OF DIAGNOSTIC TESTS

		THE REAL TRUTH[1]	
		Positive	Negative
WHAT	Positive	True Positive	False Positive
WE FIND	Negative	False Negative	True Negative

[1] Which can never be known with certainty

FIGURE 2

HOW PREDICTIVE VALUE OF A TEST CHANGES WITH DISEASE PREVALENCE

Case 1. True prevalence = 1 case/100

	THE REAL TRUTH		
	Positive	Negative	Total
Test Positive	990	990	1,980
Test Negative	10	98,010	98,020
Total	1,000	99,000	100,000

Case 2. True prevalence = 1 case/1,000

	THE REAL TRUTH		
	Positive	Negative	Total
Test Positive	99	999	1,098
Test Negative	1	98,901	98,902
Total	100	99,900	100,000

How predictive value changes with disease prevalence. Consider the cas of an excellent test that is 99% sensitive and 99% specific, and how its predictive value would change if the true prevalence of a conditio were either 1 case per 100 persons, or 1 case per 1,000 persons. Assum that 100,000 people are tested, with 1% false positives and 1% false negatives.

In the first case (true prevalence = 1/100), half of the people determined to be positive really were not; in the second case (true prevalence = 1/1,000) more than 90% of the persons who tested positive have been incorrectly classified- by a test that is 99% sensitive and 99% specific! The positive predictive value of any test becomes smalle with declining prevalence, until the point where prevalence reaches zero. Then the positive predictive value is also zero because every positive test is incorrect. Similarly, the negative predictive value will be zero if every person in a population has the condition.

In possibly life-threatening situations, such as testing individuals for HIV infection or screening blood for transfusion, additional confirmatory are usually appropriate.

Test interpretation

Validating data are often not available for diagnostic tests, or for the accuracy of specific products, laboratories, or survey teams, particularly when tests are done in smaller laboratories or under fiel conditions. Ever when perfectly performed, all diagnostic tests requir interpretation, and in addition to quality control in the laboratory (discussed in detail in a later section), the following elements must be considered:

methods of collection, preservation, storage and handling of the specimen;

the effects of other diseases (Berner, 1984; Friedman et al., 1980), or of medication, nutritional condition, age, genetic or ethnic heritage, or other patient-related factors;

the fact that the demonstrated presence of a particular organism (even if pathogenic) does not necessarily prove a causal relation to any clinical disease. For example, the finding of Campylobacter bacteria in a diarrheic stool does not of itself demonstrate that it caused the current illness.

the performance history of the particular laboratory, including its experience with the specific test in question;

Classical methods of diagnosis of infectious diseases and their causative pathogens are listed on Table 3.

PRACTICAL ASPECTS OF DIAGNOSTIC TESTS

A comprehensive evaluation of a diagnostic test involves more than just validating that the outcomes are technically correct, i.e., that the test is sensitive and specific and can distinguish between different states or conditions.

Cost

Issues of cost are always important, critically so where funds are the most limited. In the developing countries of Latin America and other areas of the world, the debt crisis has restricted funds available for social programs such as health services; therefore, all procedures including diagnostic tests must be made as cost-effective as possible.

TABLE 3

SOME COMMONLY USED METHODS,

BY DEGREE OF SPECIFICITY, FOR DIAGNOSIS OF

PATHOGENIC ORGANISMS AND INFECTIOUS DISEASES

<<more specific>>

1. Direct demonstration of physical presence of pathogen(s) in tissue, blood, cerebrospinal fluid, sputum, urine or stool;
2. Recovery of the organism after inoculation of the specimen listed in (1) into culture medium, cultured living cells or tissues, or inoculation into an animal;
3. Evidence of the presence of pathogen-specific DNA by hybridization probe;
4. Demonstration of a characteristic antigen, which is a component or product of the organism;
5. The presence of a diagnostic titer of specific antibody in blood or other body fluid;
6. Positive reaction in a skin test;
7. Identification of the causative organism in a clean insect vector permitted to feed on the patient, as used in xenodiagnosis for Trypanosoma cruzi in Chagas' disease;
8. Clinical impression of a trained and experienced observer;
9. Interpretation of characteristic image by X-ray or CAT scan;
10. Evidence of probable exposure or infection from history, travel, activities, and epidemiological association

<<more general>>

It is important to consider total cost, which is always greater than the immediate marginal cost of performing the n+1th test in a series. In addition to the obvious supplies, materials and staff, there are capital costs such as obtaining and maintaining buildings and equipment. An economy of scale is built into diagnostic tests, particularly screening procedures. Many kinds of immunologic tests are prohibitively expensive when performed only occasionaly, but might become cost-effective when performed in greater volume.

Time

Another significant element in cost-effectiveness is the time needed to perform the test and to report results. This may be crucial clinically, as it must be known quickly whether a patient's coma arose from cerebral malaria, meningitis, or other cause. In addition, rapid tests promote laboratory efficiency, reduce staff salary costs, and assist the patient and his family. A fast turnaround time may make a second clinic visit unnecessary, saving costs in time, transportation, meals, lodging, lost work or school, and the chance that the patient will not return for follow-up.

Other considerations

Hazards. Some tests that employ potentially hazardous procedures, or toxic or radioactive chemicals, may pose a risk to patients, staff or the environment. These may require costly facilities for containment or disposal and should be carefully evaluated for feasibility.

Decision analysis. Any decision involves tradeoffs. For example:

1. There is a new serologic test for a certain intestinal parasite. The test has been found slightly less sensitive and specific than the microscopic examination of a stool sample. Is it preferable to add

the new procedure to an existing battery of serologic tests if a blood specimen is to be drawn anyway, so that the money saved by not doing the stool exam could be used for another purpose?

2. Would it be better to do inexpensive, rapid, but slightly less accurate community-based screening from a mobile or fixed clinic, or more sophisticated, costly and accurate studies in a central laboratory?

Such questions can be answered only in situational terms, depending upon the the local prevalence of a disease, whether a serious outbreak must be contained, and other factors which will vary with place and time.

Relevance

There is also the important issue of relevance or clinical utility- whether the diagnostic test results contribute to improved outcomes for patients. Does it make scientific and ethical sense to diagnose a condition in the absence of effective treatment? The answer may be yes, for the following reasons: 1) from a community viewpoint, additional cases may be prevented. e.g., by behavioral changes in an individual infected with HIV; 2) epidemiologic knowledge may be gained about spatial and temporal distribution that may lead to strategies for control of the disease; and 3) from the patient's viewpoint a diagnostic finding of an untreatable disease may be useful by ruling out one or more other possible causes of his condition.

Priorities

A recent report (IUIS/WHO, 1988) on laboratory investigations in clinical immunology discussed the use of immunodiagnostic tests for infectious diseases in lesser developed countries, denoting the following as areas of high priority:

1. Simple, reliable and inexpensive immunodiagnostic tests drawing on new technologies and innovative ideas for application:
   a. to individuals in the field for diagnosis or for epidemiological screening;
   b. in central laboratories for large populations.
2. Expanded training opportunities for laboratory personnel.
3. Improved monitoring, quality control, and standardization.
4. Partnerships between institutions in the more developed countries with their counterparts in less developed countries, to facilitate cooperation and collaboration.

IMMUNODIAGNOSTIC TECHNIQUES

IMMUNOREACTIVE ELEMENTS

Immunologic diagnosis is based on the chemical reactivities of immunoglobulin proteins, produced by the host in response to an antigenic stimulus. For immunodiagnosis of infectious diseases, the reactive elements usually sought are: host immunoglobulins (antibodies), typically in the blood or other body fluids, and parasite antigens, which may be in body fluids or tissues.

All serologic techniques aim to measure the intensity of a reaction (or titer), indicating the amount of reactive immunoglobulin in the patient's body fluids. Differences in reagent concentration, composition of buffers and other chemicals, temperature, condition of supplies and apparatus, and many other factors may affect the outcome of all types of serologic tests. Discrepant results may arise from different test formats applied to the same specimen, or from the identical test repeated at different stages of infection, such as incubation, illness, or convalescence. Depending upon the evoking pathogen, immunoglobulin class, and other factors, immunoreactivity may persist for varying periods even in the face of continued infection, and after the infectious agent has been eliminated naturally or by drug treatment. Variations among different laboratories have already been mentioned, so that the interpretation of serologic tests requires considerable experience and sophistication.

There is a serious need for standardization and increased reliability of test results, which can be helped by careful attention to potency and uniformity of reagent materials, training of laboratory personnel, frequent calibration of instruments and techniques, and periodic testing of coded specimens to confirm the accuracy of results.

Related to the evaluation of host antibodies produced in response to infection is the more recently developed immunologic technology to detect circulating antigens or other products released by an infecting organism. An advantage of this type of test is that it indicates current infection, unlike serology which may indicate some past experience with the organism. A test for circulating antigen that employs a well-defined monoclonal antibody may be highly specific for pathogen identification, in some parasites even indicating species and stage. The ability to produce large amounts of standardized monoclonal antibodies is an important factor in the reliability, comparability and reproducibility of antigen detection procedures.

Other immunodiagnostic procedures have been developed to identify antigens in tissues. These depend upon the availability of monoclonal or specific polyclonal antisera for the initial reaction, followed by a procedure that provides a signal to indicat that the reaction has taken place. A variety of immunofluorescent and immunochemical methods has been devised for this purpose, as decribed below. Where parasites or microorganisms are involved, immunologic tests for antigen detection may be in competition with molecular methods such as hybridization probes, which can identify pathogens on the basic of their chromosomal or extranuclear nucleic acid base sequences.

There remains a great need for simple, reliable and inexpensive diagnostic methods, that do not require sophisticated laboratories, to identify infected individuals, and to specify the etiologic agent in infectious diseases. This need is made more acute by current efforts to develop vaccines for a variety of parasitic, vectorborne and infectious tropical diseases. Field trials of vaccine efficacy will be held hostage to diagnostic methods, which must determine the initial susceptibility of vaccinees and controls, and their attack rates of the particular infections against which the vaccine is intended. In a rapidly advancing field, new immunodiagnostic methods are tending to ever greater definition of reagents. On the immunoglobulin side, these are based primarily on monoclonal antibodies, described in more detail below, which have revolutionized certain immunoassays. On the antigen side, advances in <u>in vitro</u> culture methods have made it easier to obtain purified viral, bacterial or parasite fractions of greater diagnostic relevance. Many highly specific proteins and peptides can now be made artificially, either by recombinant DNA technology or by chemical synthesis. All of these products promise greater accuracy, if not always simplicity of use, and they can usually detect smaller and smaller amounts of their target material.

AGGLUTINATION TESTS (Kagan, 1982, 1986; Nichols et al., 1986)

One of the most basic and important of all immunological reactions is the cross-linking of antibodies and antigens in a classical chemical reaction. If either antigen or antibody is bound to any of a wide variety of small particles, these will clump together visibly in the presence the other, forming the basis of an agglutination assay.

The simplest agglutination protocol is the direct agglutination assay in which known whole organisms, bearing their own surface antigens, are mixed with an unknown serum sample. In the presence of the appropriate antibodies, clumping of the organisms will occur. The complementary assay, using serum known to contain antibodies, can be used to test for antigens in the sample. The classical Widal test is a direct agglutination test for Salmonella species. Other similar tests have high sensitivity and specificity, e.g., for malaria parasites as well as for Trypanosoma cruzi, the agent of Chagas' disease.

Direct agglutination tests are generally straightforward, but require a pure and stable suspension of organisms (or antiserum). Potential difficulties include possible autoagglutination, and the subjectivity of interpreting results.

Indirect or passive agglutination refers to the agglutination, by antibodies, of cells or particles coated with soluble antigens. For example, bentonite, an aluminum silicate, is the carrier particle in the bentonite flocculation assay used for amebiasis, ascariasis, echinococcosis, schistosomiasis, toxocariasis, and trichinellosis. Latex particles are used in various latex agglutination assays for similar infections as well as for diagnosis of HIV infection. Commercial kits are available for most of these diseases. The objective reading of average particle size by refractive index is done by instruments in such assays as particle-enhanced turbidimetric assay and particle-enhanced light-scattering immunoassays.

The use of human or animal erythrocytes as carriers forms the basis of the indirect hemagglutination assay (IHA). Here, antigens are adsorbed onto untreated red blood cells or onto cells pretreated with

tannic acid or other reagents (tanned RBCs), or antigens may be linked covalently to the RBC surface. This test is widely used in many infectious and parasitic diseases, for many of which specific kits are commercially available. Problems with the IHA test include: limited shelf-life for reagents, difficulty of standardization, and a subjective endpoint, although automated versions of the IHA test have been described.

Indirect hemagglutination inhibition is a competitive assay between soluble antigens and antigens bound to particles. Agglutination of particle-bound antigens is inhibited by presence of antigens in the sample, with a resultant increase in test sensitivity.

Among less widely used agglutination tests, immuno-electron microscopy is used sometimes to assess the level of particle agglutination at the EM level. The requirement for an electron microscope limits the application of this procedure, which is valuable for detection of certain non-cultivable viruses such as hepatitis virus, rotavirus, and Norwalk gastroenteritis viruses. Coagglutination utilizes staphylococci, which bind to the Fc regions of antibodies of the immunoglobulin G class. A mixture of staphylococci, specific IgG antibodies, and appropriate antigens causes agglutination of the staphylococci. This test has been used in diagnosis of cholera, pneumococcal pneumonia, Salmonella enteritis, typhoid fever, and streptococcal infections.

Advantages of agglutination tests. The techniques are relatively simple and are highly sensitive even though not at the level of RIA or ELISA (discussed below). Simplicity of technique, rapidity of assessment, and results that can be read with the naked eye or a light microscope

make agglutination tests useful for the laboratory or for quick diagnosis of many diseases in the field.

Disadvantages of agglutination tests. Among disadvantages of agglutination assays are their variability and difficulty of standardization, so that the same sample may yield a fourfold difference in titers in different runs. They are also relatively inefficient for the IgG class of antibodies, which have fewer antigen binding sites and therefore agglutinate less well. It is also sometimes the case that in an excess of either antigen or antibody no agglutination takes place. In addition, nonspecific interference from rheumatoid factor and heterophile antibodies can lead to false positives.

COMPLEMENT FIXATION (CF) Greenwood and Whittle, 1981; Palmer et al., 1986)

The Complement Fixation (CF) test is the oldest, and formerly the most widely employed of all serological tests. Although its sensitivity has been surpassed by more recent techniques such as RIA and ELISA, the CF test is still used in many laboratories to determine the presence of antibodies in serum. The CF test is based on the immunological principle that complement components can be activated by antibody-antigen complexes.

Complement activation is a major line of immunological defense. When IgM or IgG antibodies react with antigens, the resulting antigen-antibody complexes, which can be either in solution or on cells, activate complement factors. The resultant interaction destroys the complexes. The competition between antigen-antibody complexes in patient antisera, and antigen-antibody complexes on red blood cells, is the key to the CF test. The sample with suspected antibodies is reacted with known antigen in the presence of a limiting amount of complement

factors. Subsequently, a standard amount of sensitized erythrocytes (red blood cells which contain antigen-antibody complexes on their cell membrane) is added. Controls are run to make sure that the test antigen itself does not activate complement, and to set up a curve for correlation between the amount of antibody and the amount of hemolysis. If there are antibodies in the sample, the amount of hemolysis will decrease since complement activated by the antigen-antibody complexes in solution cannot lyse the sensitized red blood cells. Test results can be qualitative or quantitative, and may be read by color or with a colorimeter or spectrophotometer. The same test can be applied for detection of antigen as well.

The CF test has been used for detection of numerous infectious and parasitic diseases, and commercial kits are available for Chagas' disease, echinococcosis, toxoplasmosis, fascioliasis, and schistosomiasis. For leishmaniasis and Chagas' disease, the CF test may remain the test of choice.

Advantages of CF tests. The CF test has the primary advantage of good specificity, but it has gradually been displaced by newer technologies which have greater sensitivity.

Disadvantages of CF tests. The test is technically and conceptually complex, and quality control is difficult. A relatively large amount of antigen is needed. The reagents, especially the erythrocytes, are variable, and complement is unstable and has a limited shelf life. The concentrations of test reagents such as complement and hemolysin are critical, and a technician error is disastrous to proper results. Equipment such as a centrifuge and refrigeration present a problem in the field. Another difficulty is the fact that many samples have

anti-complementary activity even without the specific antibody due to presence of some lipids and immune complexes. The practical problems in equipment, technician skills, and storage conditions limit the possible use of CF in developing countries to well-equipped, specialized laboratories.

IMMUNOFLUORESCENCE (IF) OR FLUORESCENT IMMUNOASSAYS (FIA) (Burnett, 1983; Nakamura and Robbins, 1986)

The utilization of immunofluorescence has been an essential element in the immunologic toolkit since the pioneering work of Coons in the 1940s. Immunoreagents conjugated (permanently bound) to fluorescent compounds have been used in thousands of research projects as well as in immumerable diagnostic tests.

The principle of immunofluorescence rests on the observation that some molecules (called fluorochromes) absorb light of short wavelength, such as ultraviolet light, and emit a longer wavelength of light in the visible spectrum. In many cases, particularly for immunohistochemical or immunocytochemical studies of tissue sections on microscope slides, a fluorescence microscope is required, with special optics and an ultraviolet light source. The most common fluorochromes used in FIA are fluorescein, rhodamine, and 1-dimethylaminonaphthalene-5 (DANSYL). Certain other compounds are being used or developed for this purpose.

The most frequent IF test, the direct FIA, looks for antigen in the sample by incubation with fluorochrome-labeled antibody, washing away any unbound antibody, and looking for antigen-antibody complexes indicated by fluorescence in the sample. The antigen is often part of a tissue section or cell suspension, or it could be immobilized on a solid phase.

The indirect FIA uses antigen immobilized on a solid phase. After incubation with unlabeled first antiserum and washing away unbound

antibody, a sandwich is made by adding a second, fluorochrome-labeled, antibody that will bind to the immunoglobulin of the species in which the first antibody was made. A second washing will reveal the sites of the original antigen-antibody combination. The direct test is simpler and more specific, but requires a separate fluorochrome-conjugated antibody for each antigen being sought. The indirect test is more sensitive and needs only one labeled second antibody, usually available commercially, to test for the binding of any antibody derived from the same primary species. For example, a very large number of different antisera can be produced in rabbits. The binding of any rabbit-derived antiserum used as a primary antibody can be demonstrated by the use of an anti-rabbit serum made in goats and then fluorochrome-conjugated. Similarly, the localization of any mouse-derived monoclonal antibody can be made with conjugated anti-mouse antibody made in rabbits, goats, or another species.

Immunofluorescent methods are common in the diagnosis of infectious viral and bacterial diseases, and for many parasitic infections including amebiasis, Chagas' disease, echinococcosis, fascioliasis, filariasis, leishmaniassis, malaria, schistosomiasis, toxoplasmosis, and trichinellosis, and tests for ascariasis, cysticercosis, giardiasis, and toxocariasis are being developed. Commercial kits are available for Chagas' disease, toxoplasmosis, malaria, and schistosomiasis, based primarily on noncompetitive indirect fluorescent antibody (IFA) methods. The sensitivity of this test is reported to be around 90% to 95%. Fluorescent immunoassays are also widely used in rapid viral diagnosis, especially for viruses that cannot be cultured. The growing availability of specific monoclonal antibodies is helping to make viral immunoassays more sensitive and specific.

Immunocytochemistry. Both direct and indirect immunofluorescence can be used to localize specific antigens by means analogous to those described above (see also the discussion of immunocytochemistry in the section on enzyme immunoassays).

Advantages of fluorescent immunoassays. Immunofluorescent tests are simple, rapid and straightforward. With the exception of the microscope, the necessary materials are relatively inexpensive and the protocols can be readily standardized. In many cases crude antigens or unlabeled antisera can be used and immunofluorescence assays are adaptable to field use, especially for preliminary screening. More sophisticated FIA procedures, including automated processing, can be performed in reference laboratories.

Disadvantages of fluorescent immunoassays. Most FIA procedures are not as sensitive as ELISA or RIA, but are adequate for most purposes. All immunohistological fluorescence assays require a fluorescence microscope, a moderately costly piece of equipment. However, fluorescence microscopes last for many years and can be used also with incandescent bulbs for normal light microscopy. These tests are not quantitative. Even when performed by experienced personnel, immunofluorescent tests must be read subjectivity. Nonspecific reactions may confound readings, and positive and negative control slides should be run in parallel thereby increasing the time and cost of the procedures. Fluorescent reactions are transient and will fade within hours or a few days, so that slides can not be stored for later re-examination. Photographs should be made for permanent records of reactions.

PRECIPITATION AND RELATED TESTS (Kagan, 1982; Grieco and Meriney, 1983)

Precipitin tests have been part of the armamentarium of immunodiagnosis since the early days of microbiology. The numerous interconnections of antigens and antibodies may lead to an insoluble complex that precipitates in solution. When performed in semisolid substrates such as agarose gels, a variety of semipermanent immobilized precipitin bands may be formed.

The simplest precipitation test involves a solution containing antibody that is layered carefully over a solution containing antigen (or vice versa), producing a visible band of precipitation at the interface. With mixing or centrifugation the precipitate may be found at the bottom of the tube.

A formerly much used test which still has many applications is the Double Diffusion test of Ouchterlony. In this procedure, cylindrical wells are cut into an agarose substrate and filled with solubilized test materials (antiserum or antigen). Diffusion of the materials through the substrate results in visible arcs and zones of precipitation wherever complementary antigens and antibodies meet to form complexes. Various patterns of wells can be used to test the reactivity of a number of antigens against a single antiserum, or the reverse. For example, a sheet of agarose may be poured in a small circular petri dish, specific soluble antigen placed in a central well and four test antisera put in wells at 3, 6, 9 and 12 o'clock, The agarose sheet may be dried and preserved with the precipitin lines intact.

An adaptation of immunodiffusion, often done on agarose-coated glass slides, is immunoelectrophoresis (IEP), in which long troughs and circular wells are cut into the substrate. Among the many variants of

this basic procedure, components of a complex antigen can be separated electrophoretically into a number of bands. Then antibody is placed in a long trough and immunodiffusion is allowed to occur. This technique has been used to detect echinococcosis, amebiasis, cysticercosis, fascioliasis, and schistosomiasis as well as microbial diseases and normal and abnormal serum components. Also used is counterimmuno-electrophoresis (CIE) in which antigen and antibody are placed in opposite wells and caused to migrate toward each other with an electric current. IEP and CIE procedures are relatively fast and simple and require only a basic laboratory with an appropriate power supply. The CIE test has been used in diagnosis of a variety of viral, bacterial, parasitic and fungal diseases.

An example of a specialized application of simple immunoprecipitation is the circumoval precipitin test (COPT) for schistosomiasis. Schistosome eggs placed in serum from infected persons will form blebby precipitates at the egg surface, whereas in normal serum no reaction is seen. Although this test is relatively sensitive and specific, it depends upon a supply of clean, reactive eggs from animal infections, which may be time-consuming to obtain and difficult to standardize. It also requires a microscope. The subjective evaluation of a positive result may also vary between individuals.

Advantages of precipitin tests. Precipitin tests have the advantages of simplicity and low cost.

Disadvantages of precipitin tests. They are generally nonquantitative, and may have limited sensitivity. Although they have fallen out of favor in recent years, they may be appropriate for use in some field survey situations where more sophisticated ELISA tests are not feasible. The use of monoclonal antibodies in precipitin tests could greatly improve specificity.

ENZYME IMMUNOASSAYS (EIA) (Kurstak, 1985; Maggio, 1982; Landry and Fong, 1985; Houba, 1981; Burnett, 1983)

Enzyme immunoassays (EIA) constitute a group of immunodiagnostic tests that employ an enzyme as an indicator label to detect immune reactions. Since the early 1970s, EIAs have been an essential part of the immunodiagnosis of infectious diseases, and have been used also for many other purposes.

The essential element of an EIA is the linking of an active enzyme either to an antigen or to an antibody. The underlying assumption is that both the normal catalytic function of the enzyme and the immunoreactivity of the antigen or antibody remain unaltered by their combination. There are many variations depending on the number of components used, which components are labeled, and whether the labeling is done covalently or noncovalently. There is an intial incubation of the test (unknown) material with the known enzyme-linked component (such as the antibody), followed by washing away of the unbound enzyme-linked reagent, and a final incubation with a chemical substrate that produces a colored product in the presence of the enzyme. This general strategy is a powerful tool for detection of antigen-antibody reactions.

One commonly used EIA is the immunoperoxidase technique, which resembles a fluoroimmunoassay (FIA) but uses an enzyme-conjugated rather than a fluorescein-conjugated immunoreactant. Horseradish peroxidase is used as the enzyme label conjugated to the antibody. After primary reaction, incubation with the proper substrate produces an insoluble colored reaction product visible by eye or, in an immunocytochemical procedure at the histological level, under the light microscope. Compared to FIA, immunoperoxidase eliminates the need for a fluorescent microscope and allows for more sensitivity since the

enzymes can continue to amplify the reaction. The color reaction is stable and permanent, whereas immunofluorescence fades quickly. Disadvantages of enzyme immunocytochemistry with immunoperoxidase are that it is somewhat more complex to perform than the similar fluorescence assay, and that many tissues possess endogenous peroxidase that must be accounted for. This problem may be avoided when alkaline phosphatase is employed as the conjugating enzyme in an analagous procedure, in which case a different final substrate is needed.

The ELISA test: various configurations

The most important EIA is the ELISA (Enzyme-linked immunosorbent assay), in which one immune-reactive element is attached to a solid-phase surface such as plastic paddles, beads, disks, or tubes, and the other imune-reactive element is added in soluble form. The concave wells of molded plastic microplates have become standardized so that many tests, often 48 or 96, may be performed simultaneously. In addition to the basic underlying assumptions of EIAs mentioned above, all ELISA tests require the maintenance of normal immune reactivity of the immunoreagent after its attachment to the solid phase.

The term ELISA now includes a family of related procedures in addition to the basic competitive ELISA, which in some respects resemble radioimmunoassay procedures.

In the Indirect ELISA, antigen is first bound onto the solid, usually a microplate well, and the sample with the unknown test antibody is then added. After interaction under appropriate conditions, any remaining unbound antibodies are removed by washing and an enzyme-labeled anti-immunoglobin reagent is added. After a second incubation and washing, the appropriate substrate is added and enzyme activity in the well is measured by determining the degree of resultant color, either visually or with a photometric ELISA plate reader.

With this powerful technique, a single serum sample may be divided and tested for any number of antibodies, using the identical enzyme-linked anti-immunoglobulin reagent, provided only that the various antigens are bound to different wells of the microplate(s). Moreover, the immunoglobulin class (IgG, IgE, etc) of the antibody can be determined by using class-specific enzyme-linked immunoreagents, readily available commercially. This technique has been used for antibody measurement in "virtually all human and animal infections" (see Voller and Bidwell, 1986).

It may be that a pure and specific test antigen is not available, but a specific antibody, such as a monclonal, exists. In that case it is possible to attach that antibody to the solid phase first, then add a crude antigen mixture, of which only one specific kind may bind to the attached antibody. At this point the unknown antiserum is added and the reaction proceeds as described. Numerous other ingenious variations have been described.

A converse of the previous tests can be used to measure the concentration of antigens in a sample. A reference curve of color intensity is established using known amounts of attached antigen, enzyme-labeled antibody, and enzyme substrate. In the inhibitory ELISA a sample with an unknown antigen concentration competes for antibody binding with the standardized solid-phase antigen. Reduction in enzyme activity (= color intensity) is related to the amount of free antigen in the sample.

A similar test is the competitive ELISA, in which the antibody is attached to the microplate well and a reaction curve established using enzyme-linked antigen. A sample containing unknown antigen is added to

this standardized system, whereupon any free antigen in the sample competes with the enzyme-linked reference antigens for binding with the attached antibody. Again, a reduction in enzyme activity indicates the amount of antigen in the sample. A variation of this test uses solid-phase bound antigen, sample antigens, and labeled antibodies.

The sandwich ELISA involves the use of a solid-phase bound antibody (or antigen) and the same antibody (or antigen) linked to the enzyme, depending upon whether the test is looking for antigen (or antibody). The solid-phase attached antibody is used to capture the antigen in the test sample, and the enzyme labeled antibody permits measurement of the amount of antigens. It is now posible through use of monoclonal antibodies to use one attached antibody and and another enzyme labeled antibody, both of which recognize the same antigen, but in different places (epitopes), leading to a more specific but less sensitive test than the Indirect ELISA.

The Avidin-Biotin reaction

Among the variants of ELISA tests is the avidin-biotin technique used in binding enzymes to antibodies. Avidin is a substance derived from egg white, which was discovered when cats fed raw eggs developed a deficiency in the B-vitamin biotin. There is an extremely firm chemical bond between biotin and avidin, both of which can be linked chemically to either antibodies or enzymes. The addition of "biotinylated" enzymes to avidin-linked antibodies can produce a very sensitive test for antibody because one antibody unit can bind several enzyme molecules.

Requirements and problems of ELISA tests

As immunodiagnostic tests become more complex and sophisticated, the issue of quality control is more and more important. All forms of

ELISA tests demand 1) a chemically correct and properly prepared solid phase, 2) suitably diluted (or concentrated) samples of both antigen and antibody, 3) chemically active enzyme and substrate, 4) fresh diluents and buffers of the proper chemical composition, 5) a correctly timed sequence of incubations and rinses, and 6) a sensitive detection mechanism. The technical staff must be properly trained and experienced, and must know how to correct the procedures when things appear to go wrong. Standardization of the test components is imperative in order to get optimal results. Frequent verification with positive and negative controls is a necessity. Microplates and other components are different from different manufacturers, and may vary even within a single package. The attachment of a uniform, reactive layer of correctly prepared immunoreagent to the convex wells of the microplate is a complicated and exacting process. When properly done, the plates are stable and may be stored under suitable conditions for several years.

The enzyme- substrate systems used in ELISA tests must yield an unequivocal end product, readily detected and quantificable. Horseradish peroxidase and alkaline phosphatase have been mentioned, and lysozyme, glucose-6-phosphate dehydrogenase, and beta-galactosidase have also been used. Horseradish peroxidase is good for general purposes since it is cheap, readily available in purified form, and easily conjugated to the immunoreagent by several methods. In most cases a colored product is produced, but the enzymatic cleavage of some specialized substrates yields fluorescent products that can be detected at very low levels using an appropriate ultraviolet light source and sensitive detection system.

Advantages of EIAs. Enzyme linked immunoassays when properly performed can rival the sensitivity of radioimmunoassays. Advantages of ELISA over RIA include 1) a relatively long shelf life; 2) relatively simple and inexpensive equipment without the need for costly radioactivity counters; 3) avoidance of radioactivity with its potential hazards and complicated regulations for purchasing, shipping, storage, utilization and disposal; 4) a variety of presentations, attachment and linkage options, choice of enzymes and substrates, conjugation techniques, and readout methods; 5) high sensitivity with the use of monoclonal antibodies for antigen detection.

Disadvantages of EIAs. Difficulties include 1) ELISA can be complex and time consuming; 2) the poor binding affinity of some monoclonal antibodies; 3) the need for continuing checks to be certain that enzymes, substrates and immunocomponents are potent and not degraded.

Immunocytochemistry

Just as ELISA can provide qualitative and quantitative information about the antigens and antibodies present in a sample, immunocytochemical techniques can demonstrate their morphological localization. As implied by the name, immunocytochemical methods are used primarily on frozen or paraffin-embedded tissue sections, usually to localize antigens. Signal detection, carried out by the same enzymatic procedures described above, produces a permanent colored product in the tissue section to show the precise location of the antigen in question. When fluorescein-conjugated antibodies are used, a bright apple green to yellow fluorescence, visible through a fluorescent microscope, shows the same areas. However, the fluorescence fades rapidly and a permanent record can be made only by photography.

Monoclonal antibodies increase the specificity of immunocytochemical procedures and may demonstrate pathogens when other methods fail; for example, a patient considered negative for HIV infection by ELISA and western blot was found positive by immunocytochemical staining with a monoclonal antibody to a specific viral antigen (Duggan et al., 1988).

IMMUNOBLOTS or WESTERN BLOTS (WB)

The method of immunoblotting is a powerful analytic tool much used in the research laboratory. It has found its greatest diagnostic application in confirmatory tests for HIV antibodies following ELISA. The general logic of this method is similar to that described for ELISA tests, except that the antigen is first fractionated and immobilized on a special type of paper.

To make an immunoblot by the most common process, it is necessary first to prepare a polyacrylamide gel separation of material containing a known antigen. In this method the complex mixture of proteins and peptides is placed in a well cut into a slab gel, and then separated into bands according to molecular weight when an electric current is passed through the gel. Subsequently, a piece of nitrocellulose paper is pressed firmly against the flat surface of the gel and, under the influence of another electric current, the separated protein and polypeptide bands move out of the gel and onto the paper, to which they become firmly bound. If the paper with the still-invisible bands is then incubated in a solution containing serum with antibodies against materials on one or more of the bands, binding will take place at the relevant places on the paper. The location of this binding can be

visualized by use of an appropriate second antibody conjugated to an enzyme, usually horseradish peroxidase or alkaline phosphatase. This is followed by incubation in the enzyme substrate, which deposits a colored product at the site.

In practice, a broadly blotted nitrocellulose paper may be cut into numerous parallel strips 2 or 3 mm wide, each bearing an identical series of latent bands. Each strip may be incubated in a separate small well in a tiny amount of diluted serum from a different person, offering a considerable saving in materials. In practice, an immunoblot for HIV is considered positive if there is reactivity in at least one gag gene-encoded band (at 17, 24 or 55 kD) plus one env gene-encoded protein (at 41, 120 or 160 kD). The variety of potentially reactive bands renders the interpretation of immunoblots more difficult.

It is possible also to use the identical techniques to identify antigens in a sample (e.g., of homogenized tissue) when a known positive serum or a monoclonal antibody of defined specificity is used in the first incubation step.

Advantages of immunoblotting. The fractionation of the sample and incubation with antiserum permits localization of antibodies to specific components, e.g., to fractions of known molecular weights and can therefore differentiate different specific antibodies that would all give an indentical reaction by ELISA or immunocytochemistry. The preparation of numerous identical strips can help to standardize the reaction.

Disadvantages of immunoblotting. The test is very expensive and requires specialized apparatus and chemicals. Preparation of the blotted nitrocellulose paper or strips is time-consuming and expensive.

Immunoblotting must be carefully done by well trained technicians. Reading endpoints is subjective and false positive reactions are not uncommon.

RADIOIMMUNOASSAY (RIA) (Larsen and O'Dell, 1986)

Radioimmunoassay is a powerful and technically demanding procedure used to determine levels of hormones, some drugs, and many antigens. The technique was introduced in the mid 1950's by Berson and Yalow, who studied antibodies to insulin in diabetics.

The most common configuration of this test is the competitive RIA, which involves a known amount of radioactively labeled antigen, an unknown amount of unlabeled antigen (which is being determined), and a standard amount of antibody. The antibody used can be from polyclonal antiserum or monoclonal. The amount of radioactivity emitted by the labeled antigen alone is compared with the amount of radioactivity emitted when the unknown (unlabeled) antigen is added, permitting a quantitative measurement of the amount of unlabeled antigen is in the sample.

The procedure is not so simple, however, because antigens in serum may exist in free or bound states, and for accurate determinations it is necessary to separate bound and unbound antigens. This may be done by precipitation of some antigen-antibody complexes out of solution, through the use of various solvents in which free antigens, but not immunoglobulins or complexes, are soluble, and by other means. A common separation technique is the solid phase competitive RIA, in which the antibody is made insoluble by physical adsorption to polystyrene tubes or wells or by covalent binding to particles.

Another type of RIA measures the level of antibody, not antigen, in the sample. Here, the known antigen is solubilized by binding to a solid phase such as tubes or wells or particles, and then the sample serum is added and any specific antibody present will bind to the immobilized antigen. The bound antibody is detected and quantified with a radiolabeled anti-immunoglobulin which attaches to the bound antibody.

Advantages of RIA. Radioimmunoassay is a highly sensitive procedure. Commercial kits can detect up to one picogram of an antigen, making RIA especially valuable for monitoring various physiological functions, determination of the serum level of drugs, and measurement of hormones. Another advantage of RIA is that it is relatively simple and straightforward to carry out once the reagents and materials are available.

Disadvantages of RIA. For use as a diagnostic technique in developing countries, RIA has many problems. It may be far more sensitive than is really needed for a diagnostic determination. More importantly, the method is extremely expensive, and requires the use of radioactive isotopes. The most stable isotopes used are ^{125}I and ^{131}I which have half-life of 57.5 days and 8 days, respectively. Both of these isotopes of iodine emit gamma rays and hence are relatively dangerous. Beta emitters such as ^{3}H or ^{14}C, which have very long half-lives, also have low rates of emission. Quantitating the results requires the use of a complex and properly calibrated gamma counter for gamma emitters, or scintillation counter for beta emitters. In either case highly trained technicians are needed to do the tests and to maintain the equipment. The acquisition, shipment, use and disposal of the raiochemicals brings up legal and

environmental issues. The instability of the reagents as a result of natural decay of the radioisotope necessitates frequent replenishment of the radioisotopic materials.

Although RIA has been used to detect antibodies to many microorganisms and parasites, and is the method of choice in detection of hepatitis antigens, its problems preclude the use of this technique as a routine test in the field in developing countries. However, RIA does have a role to play in research in university or central diagnostic laboratories that contain the necessary facilities for the storage, centrifugation, measurement, and disposal so vitally needed in RIA; in the field, however, the availability of other tests such as ELISA and Immunofluorescence render RIA impractical.

OTHER IMMUNODIAGNOSTIC TESTS

Thin layer and diffusion-in-gel methods. Since the 1930s, it has been known that antigen-antibody reactions can occur on the surface of a solid or within gel media. Recently, these methods have been revised and improved: their relative simplicity and speed suggest promise for field application in tropical countries for the diagnosis of infectious diseases.

In the method of Giaver (1973), a glass slide is coated with indium particles which produce a dark brown color due to light scattering. The slide is then coated with antigen, and the sample antiserum is added. After washing, the color of the slide would change because the antibody-antigen complex would increase the thickness of the surface layer and alter its light-scattering properties. A second visualization technique is the vapor condensation on surface spot technique (VCS). Here, antigen is coated on parts of a plain glass slide, whose uncoated areas are covered with serologically unrelated proteins. The sample

antiserum is applied, incubated in a humid atmosphere for 60 minutes, rinsed, dried, and stored upside down in a humid atmosphere (Elwing et al., 1977). Results are read by looking at the condensation pattern of water vapor. A variation on this technique employs an anti-immunoglobulin prior to the visualization process which leads to better resolution; this is called the reinforced VCS.

In spot thin layer immunoassay (Spot-TIA), a polystyrene surface is used to adsorb antigens. Serial dilutions of sample antiserum are applied as spots to to the coated plate, followed by VCS. A pattern of condensation indicates a positive reaction (Elwing et al., 1977). One possible disadvantage of this technique is the fairly high concentration of coating antigen required. The Spot TIA has been tested for the detection of Entamoeba histolytica, Schistosoma mansoni, diphtheria and cholera toxin, E. coli, pneumococci, and H. influenzae.

Gel diffusion techniques, including the classic Ouchterlony and immunoelectrophoresis, have been available for decades. A recent modification is the Diffusion-in-Gel TIA (DIG-TIA). In this technique, the antigen is adsorbed to the plastic surface of a petri plate, into which a gel is poured. The sample antiserum is put into holes cut in the gel, and the antibodies diffuse radially into a concentration gradient which depends on their initial concentration and reactivity with the antigens. Visualization is carried out using the VCS technique. Sensitivity for the detection of antibodies was reported at

1-2 mg/l, the same as for Spot TIA, but can be increased tenfold with the reinforced reaction (Elwing & Nilsson, 1980). DIG-TIA is quick and simple, and does not require any particularly sophisticated equipment. It has been used to analyze herpes simplex virus, S. mansoni and E. histolytica. Its level of sensitivity, like that of Spot-TIA, still leaves much to be desired, but with more purified antigens and other improvements, tests of this general kind may be relevant to field work in tropical countries.

The diffusion-in-gel principle has been applied also to the ELISA test. As with DIG-TIA, antiserum in gel is allowed to diffuse over an antigen-coated plastic surface. However, visualization is accomplished by adding enzyme-conjugated anti-immunoglobulin antiserum which will bind to the antibody of the antibody-antigen complex. After enzyme substrate is added, the color intensity of the reaction is proportional to the quantity of antibody-antigen complexes, and the only measurement which needs to be made is the diameter of the zones. The technique is relatively uncomplicated and has a sensitivity about the same as for a reinforced TIA (Elwing & Nygren, 1979). Compared to conventional ELISA, DIG-ELISA is reported to be simpler, to require no dilution, and to be quantifiable without a spectrophotometer. However, a relatively large amount of coating antigens is necessary.

Serum absorption in filter paper

A recurring problem with immunodiagnosis of infectious diseases in the field is the problem of specimen collection and transport to a place where tests can be done. Many studies have employed filter paper to absorb blood, obtained from the fingertip in adults or the earlobe

in young children, eliminating the technical and cultural difficulty of venipuncture (Matthews, 1981). The dried filter papers can be stored in a cool and dry environment away from insects for several months. In the laboratory, the blood spot can be eluted from the filter paper, or various immunodiagnostic tests can be done right on the paper. There is some loss of antibodies, especially at low titers, and only two to four tests can be done on a single blood spot. Alternatively, elution can be bypassed in a DIG procedure by using the paper filter as the source of diffusion (Nilsson et al, 1985). The use of filter paper discs has important logistical implication both for currently employed diagnostic tests as well as new tests still in the developmental stage.

Among other recently developed immunodiagnostic tests is the Visible Immunodiagnostic Assay (VIA). Antibodies are coated onto dipsticks which are then placed in the sample to pick up antigens. The dipstick is washed and then incubated in a suspension consisting of latex microspheres covalently linked to antibodies which will attach to the bound antigens. The result is a color change on the dipstick. The test can be done in 30 minutes and is reportedly as sensitive as an ELISA but more stable since no enzyme component is needed. It is also possible theoretically to test for two antigens with the same dipstick (Klausner, 1987).

ANTIGENS

Highly specific antigens may be obtained from the organism in question by various means such as: extraction from specimens chemically or by electrophoresis and removal of the appropriate band from the gel; complexing with a specific antibody added to a suspension or homogenate

of the organism followed by capture of the antigen- antibody complex in an appropriate column and final separation and elution of the antigen; etc. In general the amount of pure antigen recovered is low and these procedures are more suitable for the research laboratory than for production-scale processes. Advances in *in vitro* cultivation techniques for malaria, schistosomes, and other organisms may help to make material available for processing. The development of recombinant DNA techniques makes it at least theoretically possible to obtain a wide variety of highly pure complex antigens by recovery and separation from the products of *Escherichia coli*, yeasts, or other expression systems which can be grown in large volumes in fermentors.

MONOCLONAL ANTIBODIES: USE IN IMMUNODIAGNOSIS

(McDade, 1985, Payne et al., 1988; Tam et al., 1985)

A severe limitation in the use of immunodiagnosis for the detection of infectious disease pathogens lies in the antibodies used in the tests. Conventional polyclonal antibodies were often insufficiently specific, and they could not be produced in a large, constant and standardized supply. The development of monoclonal antibody techniques has helped to address these and other problems, and enhances the diagnostic tests that we already have.

Prior to the development of monoclonal antibodies, antisera used in immunodiagnostic tests usually came from animals that had been inoculated with the desired antigen and then selected for good antibody response to that antigen. Often rabbits were used, but antisera were also harvested from goats, swine, horses, and other mammalian species. This led to antisera which varied in specificities, combining avidities, and titers depending on the genetics, history and idiosyncrasies of the particular animal used, as well as with the evoking antigen, interval to bleeding, and subsequent handling of the blood and serum. Each batch was different, even in consecutive bleedings from the same animal, and when that batch was gone there was no more. The cumbersome and labor intensive process of antisera collection resulted in a limited amount of available nonstandardized polyclonal antisera, whose characteristics and quality affected the

test results. Consequently, immunodiagnostic tests suffered from both the technical and economic points of view.

Starting in the early 1970's, researchers began to make headway in the search for a more constant source of specific antibodies. It was recognized that multiple myeloma, a cancer of antibody-producing cells, led to the secretion of a homogeneous immunoglobulin called myeloma protein which appeared to be antibody molecules. Starting in the mid-1970s, Köhler and Milstein fused such myeloma cells with spleen cells sensitized to sheep erythrocytes and obtained hybrid cell lines (= hybridomas) which secreted anti-erythrocyte antibodies.

This knowledge eventually lead to the development of monoclonal antibody production. Myeloma cells are created with a deficiency in hypoxanthine-guanine-phosphoribosyl transferase (HGPRT). These are fused in the presence of polyethylene glycol with spleen cells which have been sensitized to the antigen in question. The results are various fused cell combinations, one or several of which have the desired myeloma-spleen makeup. By culturing the resultant cells in a selective medium with hypoxanthine, aminopterin, and thymidine (HAT), the myeloma-spleen hybrid has a growth advantage over the parental cells since the myeloma cannot grow without HGPRT in the presence of aminopterin (no purine synthesis from hypoxanthine or de novo since aminopterin blocks this), while the spleen cells grow very slowly and can be separated from the hybrids. The hybrid cells that are secreting the desired antibody can be then selected out, cloned, and grown as a cell line by conventional procedures of tissue culture.

Advantages of monoclonal antibodies. A monoclonal antibody is really a

chemical reagent consisting of a protein that binds with exquisite specificity to a particular target chemical configuration. As such it can be used to identify highly specific targets in a test specimen, provided that some sort of signal detection mechanism is included in the test procedure. The clinical laboratory therefore has better control over its procedures through improved uniformity of antibodies used in diagnosis. Moreover, purification of the original evoking antigen is less crucial than in the case of polyclonal antisera, so the cost in time and money for this step may be reduced. Mice are generally used in the production of monoclonals,sparing the expenses of maintaining larger animals. In sum: the main advantages are the absolutely defined specificity of binding, and production in theoretically unlimited volume.

Disadvantages of monoclonal antibodies. Although the hybrid cell line is theoretically permanent and can yield an unlimited quantity of antibody, there are many technical problems. The process of establishing and maintaining the cell line is labor intensive, costly, and complex. Several months are usually needed before the cells can be characterized and stabilized. In addition, the equipment and material needed for the procedure is expensive. Fetal calf serum, a costly, undefined, and variable material, is generally required for the cell culture. Undesirable microorganisms such as mycoplasmas can be introduced with the serum unless rigid quality control is exercised. Once the cell line is established, it may change in nature, stop producing the antibody, or exhibit other technical difficulties for no apparent reason.

For application in immunodiagnosis, monoclonal antibodies may also present some problems. As they do not necessarily have the same

properties as the pool of antibodies in complex polyclonal antisera, immune interactions between monoclonal antibodies and antigens are not identical to those between antisera and antigens. A second problem comes from the almost exclusive reliance on murine (mouse-derived) monoclonal antibodies, which may not recognize the same antigenic determinants as human antibodies. (This characteristic is true of all antibodies derived from animals.) Although some human monoclonal antibodies have been produced, a general lack of a consistently high-fusion human myeloma cells has limited this technology.

A more immediate disadvantage of monoclonals has to do with decreased avidity; i.e., they commonly do not bind as strongly to antigens as do antibodies in polyclonal sera. The decrease in avidity presents a problem primarily in enzyme-linked immunoassays which demand antibodies with high avidity. Therefore monoclonal antibodies may be lacking in sensitivity.

Finally, monoclonal antibodies may be simply too specific to be of practical use in some applications. They may recognize only a portion of a large molecule, which for some reason may not be present, or exposed to interaction, in some specimens. For agents with varying antigenic determinants, a monoclonal antibody is more likely to give a negative result than a polyclonal antiserum.

> MAbs have been used to a significant degree only in selected segments of laboratory tests. The overall impact has been less than that predicted several years ago. For example, because of both sensitivity and specificity problems, implementing MAbs in tests for detecting infectious disease agents has been much more difficult than anticipated. Although MAbs are becoming more widely available from a variety of suppliers, both quality and price vary considerably. Moreover, commercial production of MAbs for highly specific applications may be unpredictable because of cost. (Payne et al., 1988)

Despite these disadvantages, monoclonal technology has much to offer to the field of routine immunodiagnosis as well as research, as many investigators (including Payne et al.) have pointed out. Once a cell line has been established, the cost of production of monoclonal antibodies is not prohibitive. The potential volume of antibodies available over the useful life of the cell line means that much of the start-up costs can be recovered, provided that there is sufficient market demand for the material. It is essential therefore to be selective in the kinds of monoclonals produced, as the same amount of work is involved in developing a hybridoma cell line for a widely used product as for a monoclonal antibody of use only in research. The issue of commercial production is dealt with in a later section.

Monoclonals are becoming more widely used in the field, particularly for diagnosis of AIDS and hepatitis, but thousands of monoclonal antibodies have been produced against antigens of all kinds. They are employed most often in fluoroimmunoassays and ELISA. Many monoclonal antibodies are marketed to distinguish different types of T-lymphocytes for diagnosis of some autoimmune, malignant and infectious diseases. In addition, numerous tumor-specific monoclonals have been made to diagnose leukemias and other types of cancer cells. For example, a specific application is in the identification of carcinoembryonic antigen which is present in colorectal cancer or chorionic goonadotropin found in testicular cancer.

An interesting application of monoclonal antibodies is in tumor imaging, in which radioisotopically-labeled monoclonals are used to identify certain tumors which are then visualized by advanced detection methods.

DNA HYBRIDIZATION PROBES IN THE DIAGNOSIS OF INFECTIOUS DISEASES

(Guatelli et al., 1989; Landry and Fong, 1985; Pollice and Yang, 1985)

Although not basically immunologic, hybridization probes must be mentioned in a discussion of modern diagnostic methods. Although the idea of hybridization for diagnosis dates from the early 1960s, practical applications have blossomed only in recent years. The application of hybridization probes is probably the most powerful new tool for diagnosis not only of infectious diseases but for malignancies, genetic diseases, and certain forensic applications as well. Kingsbury (1987) has commented that "there is a subset of diagnostic applications for which DNA probes are uniquely suited. Their foremost application, for which there is little competition from biochemical or immunochemical tests, is in epidemiological investigation in which material is either collected in field studies or collected and saved for months or years prior to screening. . . . Another application to which DNA probes are uniquely suited is in the detection of microorganisms, like the mycoplasmas, which grow poorly or not at all on laboratory media, and which show a wide antigenic variation." He has listed the following applications of DNA probe diagnostics for infectious diseases:

Mycoplasmas; bacterial endotoxin genes; latent viruses such as herpes, CMV, HIV; human papilloma viruses; chronic hepatitis B in liver tissue; direct identification of antibiotic resistance genes in bacteria; detection of virulence determinants such as the virulence plasma in Yersinia pestis; Campylobacter in stool; Plasmodium falciparum.

Despite these observations, this method as currently configured has severe limitations, particularly cost and the need for highly specialized laboratory and human resources, before it can be considered for any but experimental use in developing countries.

Unlike most classical diagnostic tests, nucleic acid hybridization probes rely upon the firm mutual binding of complementary DNA (or RNA) sequences found in all cells and are for the most part independent of immunology. However, some newly developed detection techniques (described below) utilize c ·yme-conjugated specific antibodies. Nucleic acid hybridization methods are useful in detection and identification of viruses, as well as for pathogenic bacteria and their plasmids which may control pathogenicity, toxin production and other characteristics of clinical and epidemiologic importance. Hybridization can even detect certain viral nucleic acid sequences that have become integrated into the genetic apparatus of various kinds of cells.

Hybridization procedures utilize specifically created probes, whose construction starts with the isolation of a piece of DNA (i.e., a unique genetic sequence) from a cell, bacterium, or viral particle of the particular type to be tested for. To do this, investigators use very specific enzymes (restriction endonucleases) which will cut the long strands of DNA at specific sites, determined by the DNA sequence and the particular endonuclease enzyme used. Electrophoresis is used to separate the fragment with the desired genetic sequence from the others.

The fragment of DNA thus obtained is then copied many times. The first step in amplification is insertion of the test sequence into the DNA of a larger unit (replicon, or cloning vector), which has been cut and treated to receive it. The test DNA sequence merges into the vector DNA with the assistance of another enzyme, DNA ligase. Another genetic element that confers resistance to an antibiotic had previously been incorporated into the cloning vector, and will be used in a subsequent step as described below. Plasmids are used as vectors of a sequence up to about 6,000 bases long, bacteriophage viruses can accept sequences of about 20,000 bases, and cosmids (plasmid and cos sites from lambda phage) can take insertions as big as 45,000 nucleic acid bases.

After the cloning vector, carrying the spliced-in test sequence, has reproduced thousands of times, it is introduced into a bacterium such as E. coli, a yeast or other cell via transformation (the following descriptions refers to E. coli). To assist in identifying those bacterial hosts in which the cloning vector has been introduced successfully, the E. coli are plated onto media containing an antibiotic. Those bacteria containing the cloning vector will survive and proliferate, each cell yielding a copy of its own genetic material as well as that of the cloning vector containing the spliced-in DNA sequence. The bacteria can then be distrupted, their DNA extracted, and the original DNA fragment, now present in numerous copies, can be excised and collected with use of the same endonuclease enzyme used previously, and then separated by electrophoresis. These identical fragments, pooled together, labeled with a radioactive isotope or with

another signal marker, are now called a DNA hybridization probe. The most common procedure for hybridization involves a sample whose own DNA has been denatured (i.e., the two strands separated) and attached to a solid phase such as a nitrocellulose membrane or a glass slide. The labeled DNA probe is denatured by heating, and poured onto the sample. After incubating under specific conditions for a certain time, the preparation is washed to remove unreacted probe. Any probe that attached to the sample through complementarity of their DNA sequences can be detected by means of the particular label used. For example, a radioactive probe can be localized by darkening of photographic film placed in contact with the preparation (in the dark) and then developed normally.

A special form of hybridization, developed by E.M. Southern in 1975, has come to be called a Southern Blot. In this procedure the sample is cleaved by endonucleases into fragments which are separated by electrophoresis and then denatured with NaOH. The denatured DNA is transferred (blotted) electrically to a nitrocellulose membrane, then thoroughly dried. Hybridization is performed as described above. Bands to which the probe has attached are revealed by the standard detection methods such as photographic film.

Certain technical problems limit the usefulness of this hybrdization procedure. For example, RNA does not bind to the nitrocellulose filter and thus RNA:DNA hybridization cannot be carried out in this way. Small fragments of DNA do not bind very efficiently, and perfect hybrids without single-stranded tails are lost because the nitrocellulose membrane binds only single stranded DNA. Various

ingenious means have been devised to overcome these problems.

Hybridization probes and blots on nitrocellulose membranes have been used technique has been used to detect many kinds of viruses and also differentiate between genetic strains of bacteria such as those causing diarrheal disease or gonorrhea.

A related DNA technique is *in situ* hybridization, which permits the detection of DNA sequences directly in histological samples. Tissue biopsies or similar samples are fixed and sectioned by standard techniques, and hybridization probe in solution is added to the cut sections as described above. The precise anatomical location and cell types containing the DNA sequence under study are thus revealed. For example, liver cells infected by hepatitis virus can be differentiated from uninfected cells. In a variant of this procedure, red blood cells containing malaria parasites can be identified on a blood smear, without the need for conventional staining and microscopy.

A commonly used technique is spot blot hybridization. A combination of southern blotting and colony hybridization, dot blotting requires less genetic material than the parent methods. Material may be spotted onto nitrocellulose paper before or after cell lysis and DNA denaturation. In the "dot blot" variant, a filtration apparatus is used to apply the sample as a uniform discrete spot. This fast and simple technique does not require electrophoresis, restriction enzyme analysis, size fractionation, or DNA transfer. The actual hybridization is similar to previously described methods, and its simplicity makes it useful for diagnosis of infectious diseases in the field. The test has

been used for many viruses including herpes simplex, varicella zoster (VSV), cytomegalovirus (CMV), Epstein- Barr (EBV), adenovirus, hepatitis B (HBV), papovavirus, and others, especially those that are difficult to isolate. A spot hybridization can be done within 2 days rather than the 4 to 14 days that may be needed for the far more elaborate diagnostic cell culture.

Spot hybridization has been performed on blood smears for Plasmodium falciparum, with a detection level of 0.0001% infected erythrocytes with use of a radiolabeled RNA probe. The application of spot hybridization to many other diseases is under study and new reports appear regularly.

Problems of hybridization probes

The routine use of hybridization probes in the diagnostic laboratory must wait until certain problems can be overcome. The first is the current cost of the procedure. It has been estimated (Kingsbury, 1987) that the cost must be reduced ten-fold before DNA hybridization can be competitive with immunodiagnostic techniques using monoclonal antibodies. A second problem is the need for certain equipment, particularly a baking oven, which limits use in the field. There is also the problem of time needed for hybridization, including several days for incubation, baking, and autoradiography. The major inhibitor of DNA hybridization as a routine tool is the need for radioactivity. There are formidable difficulties in shipping radioisotopes, particularly across national borders, and special training and conditions are required for their safe use. The detection of radioactivity requires autoradiography, which is time consuming (up to

2 days) must often be done at low temperature (-70 C) with an intensifying screen, and requires at least minimal photographic darkroom facilities for development. (The ^{32}P label most commonly used at present has a half-life of only about two weeks, presenting both good and bad features. This relatively rapid decay lightens somewhat the problem of disposal, but also reduces the stability of the reagent.

Because of the inherent problems with radioactively labeled probes, much research has been done on finding more acceptable methods of signal detection. Among these are the use of fluorescent dyes and enzyme coupling, but these often have high background, low sensitivity, and are subject to hazards such as enzyme inactivation. The most promising label at present is biotinylation, or conjugation of the nucleotides with biotin. After incubation of the denatured nucleic acid strands, the occurrence of hybridization has is detected by the addition of streptavidin linked to 1) an enzyme such as alkaline phosphatase or horseradish peroxidase, or 2) a fluorescent dye such as fluorescein. The conjugated streptavidin binds avidly to the biotin on the probe, and the combination is visualized by 1) addition of an appropriate enzyme substrate, or 2) use of an ultraviolet light source such as a fluorescent microscope. Such chemical reactions are more rapid than autoradiography and avoid the problems inherent in the use of radioactivity. In addition. the biotynilated probe is far more stable than ^{32}P and can be stored in a freezer for years. At its best the sensitivity of a biotinylated DNA probe may be comparable to that observed with ^{32P}-labeled DNA, but many reports show a 5 to 10 fold reduction in sensitivity. Nevertheless, given sufficient test sample

such sensitivity may be adequate for routine clinical diagnostic use. Although issues of specificity and backround remain to be resolved, biotinylated probes or their technical relatives have promise of providing workable detection systems to allow DNA hybridization to be used in the diagnosis of infectious diseases. A recently commercialized detection method involves enhanced chemiluminescence, a method in which unlabeled single-strand nucleic acid probe is added to labeling solution in the presence of horseradish peroxidase, and then fixed with formaldehyde. The enzyme-labeled probe is hybridized overnight with the blot, and then incubated in a detection reagent that elicits a peroxidase-catalyzed oxidation of a chemical (luminol) that gives off light, which is detected on photographic film.

Another nonradioactive alternative to biotin-avidin consists of a commercially available kit which also uses horseradish peroxidase, but the enzyme is conjugated to specific detection antibodies and yields a colored product with appropriate incubations and washings.

Advantages of nucleic acid hybridization. When properly performed, this method provides highly specific identification of organisms at the genetic level, making unnecessary any examination of phenotypic expression. Provided that sufficient material is available the method is applicable not only to patient-derived specimens but also to foods and other materials that may harbor the pathogen in question. As the chemically stable nucleic acids may survive the death of the organisms, hybridization procedures may work even in the absence of living pathogens and may be applied in situations where *in vitro* culture would be impossible.

Disadvantages of nucleic acid hybridization. As mentioned, the procedure is very costly, highly complex technically, and requires a well-equipped and modern laboratory with the proper reagents and trained technicians. For detection of radioactive or chemiluminescent signals, a photographic darkroom or equivalent is required.

A further problem with DNA hybridization is that although it is highly specific it is relatively insensitive so that a large number (at least tens of thousands) of copies of the target DNA sequence may be required for ordinary signal detection by radioisotopically labeled DNA, and perhaps tens of millions for colorimetric labels.

NUCLEIC ACID AMPLIFICATION

Enzymatic DNA amplification procedures, particularly the polymerase chain reaction (PCR), have become very popular (Guatelli et al., 1989). The most commonly used method (in 1989) employs a DNA polymerase enzyme derived from the thermophilic bacterium Thermus aquaticus, commonly called Taq. This enzyme can cause multiple duplication and reduplication of DNA sequences originally present in numbers too few to be detected, resulting in a large amount of material available for hybridization or other manipulation. Amplification of a million-fold is possible wiothin a few hours. Other amplification systems, employing for example the enzyme Q beta replicase, have also been described.

Although some viral pathogens such as HIV-1 are present in the blood in low copy number (10^3/ ml), it is considered premature to

apply PCR as a routine diagnostic tool. However, "the future role of sequence amplification in clinical viral diagnostics promises to be important" (Guatelli et al., 1989).

It is likely that nucleic acid amplification through the polymerase chain reaction, or a similar method, will become a major diagnostic technology in the United States and other wealthy countries. Although by its nature it will never become truly simple or inexpensive, it is possible that PCR will find application in the detection of significant infectious disease agents in developing countries.

The rapid progress in this field threatens to make any printed comment obsolete when read, and it may not be long before relatively inexpensive, safe and simple hybridization tests become available for diagnostic and epidemiologic studies of infectious diseases. However, it remains to be seen whether these will ever become practical for routine use in developing countries.

SCREENING OF DONATED BLOOD

The screening of blood for transfusion has become an important issue in all countries. Although most attention has been given to the human immunodeficiency and hepatitis B viruses, which are clearly the most important pathogens, many other agents are, or could be, transmitted through transfused blood or blood products (Table 4). Some agents, such as HTLV-1, the presumed cause of adult T-cell leukemia/lymphoma and some other conditions, are transmitted only within the cells. Some others may be found also in the plasma.

The risk of disease transmission is correlated with:

1) the frequency of the infection among the donor population;

2) The intensity of viremia or parasitemia in the donor

3) the probability that the pathogen will remain infectious through blood processing, storage, and handling procedures;

4) the effectiveness of screening procedures in detecting contaminated units; and

5) the susceptibility of recipients to infection with the pathogens;

6) the proportion of infected persons that eventually develop clinical disease.

Data on transmisson of diseases by blood transfusion are lacking for most countries, in part because of the lengthy interval between receipt of the transfusion and the onset of clinical disease. In the case of AIDS, this interval averages 4.5 years for adults and 2.4 years for

Table 4

Pathogens Transmissible Through Blood Transfusion

Viruses

Cytomegalovirus (CMV)

Epstein-Barr virus (EBV)

Hepatitis B virus (HBV)

Hepatitis delta virus (HDV)

Non A-nonB hepatitis viruses (NANBHVs)

Human immunodeficiency virus (HIV-1) (also HIV-2??)

Human B-cell lymphoma virus (HSV6)??

Human T-cell leukemia viruses (HTLV-1 = ATLV; HTLV/2)

Other viruses: herpes viruses; serum parvovirus-like virus??

Protozoa

Plasmodium of several species (malaria)

Toxoplasma (toxoplasmosis)

Trypanosoma cruzi (Chagas' disease)

Other protozoa, e.g., *Babesia*?

infants and children (Dodd, 1988). The incubation period for HTLV-1 is significantly longer. The number of transfusion-associated AIDS (TAA) cases in the world is not known, but the United States alone is believed to have more than 800 (Kühnl et al., 1989), accounting for 2.4% of adult cases and 13.9% of pediatric cases by 1988. About 1% of all U. S. cases were associated with receipt of clotting factor concentrates by hemophiliacs (Dodd, 1988), but in other countries the proportion may be much higher, e.g., 5.2% in the Federal Republic of Germany (Kühnl et al., 1989). The association of hepatitis B with parenterally administered human blood products has been well reviewed by Seeff (1988).

Some agents, such as cytomegalovirus and Toxoplasma are very widely distributed in most populations, and therefore their association with transfusion is often difficult to document. However, pathogens rare in certain countries can nevertheless be transmitted there through transfusion. For example, many cases of HIV infection in hemophiliacs have been traced to concentrated clotting factors imported from other countries. In the United States, several cases of Chagas' disease have been identified in residents with no history of travel to endemic areas, presumably from blood donated by immigrants from Central and South America where Trypanosoma cruzi is abundant. Quartan malaria due to Plasmodium malariae has been transmitted in New York from an asymptomatic blood donor who had emigrated from Greece several decades prevously and had never returned.

It is clearly not feasible to test each unit of donated blood for every organism that may conceivably be present. In regions endemic for malaria or Chagas' disease, such screening may be warranted. In other

areas, donors having certain risk factors (e.g., male homosexuals, intravenous drug users, immigrants from endemic areas) may be rejected or subjected to additional testing.

Methods of screening

Diagnosis is based on 1) detection of the agent or its component parts, or 2) detection of specific antibodies to the agent. Screening tests for blood banks should be relatively simple ("user friendly"), rapid, inexpensive, reliable, and if possible should be automated. To detect hepatitis B surface antigen (HBsAg), enzyme immunoassays and radioimmunoassays are employed, as well as a special test called reverse passive hemagglutination.

The case of HIV (HIV-1) testing

Extensive serological tests were introduced into blood banking in 1985 when the nature of the threat from AIDS became apparent. So-called first generation enzyme immunoassay (EIA) tests, based on disrupted whole virus particles, (viral lysate) are used widely to screen for antibodies in serum. The presence of a variety of cellular debris and other extraneous materials in such tests has been cited as the cause of reduced predictive power, and more than 95% of EIA-positive specimens are found falsely positive by costly confirmatory western blots. These are often beyond the financial capacity of developing countries. Second-generation screens are based on a similar principle but utilize viral antigens produced by recombinant DNA rather than those derived from viral particles. Laboratory and field studies of several second-generation test products show sensitivities and specicities equal to or better than first-generation ELISAs plus western blots (van Brunt, 1988). The numerous other configurations of HIV antibody tests demonstrate the rapid changes in technology and volatility of the

market. HIV antigen tests, based on monoclonal antibodies, have also been devised.

Many manufacturers have entered this large market, with different kinds of tests, including latex agglutination, enzyme-linked immunoassay, immunoblotting, and other configurations, many of which provide comparable results. The essential elements in a successful test to be used in developing countries include predictive value, cost, ease of use, and rapidity of results. In some countries of Africa the average time interval between donation and transfusion of blood is said to be about 30 minutes, requiring an extremely fast turnaround time for HIV testing. Several companies have introduced simple procedures that give answers of acceptable accuracy in about five minutes. Additional tests are being announced with some frequency. Most of these procedures require integrated development of the immunoreagents together with the beads, particles, plates, readers and other necessary apparatus, demanding substantial financial investment and human expertise. Therefore small biotechnology companies may be expected to be relatively noncompetitive in this market unless they can demonstrate a clearly superior product. Tests are also available for viral DNA using polymerase chain reaction and hybridization technology.

Tests may vary in their apparent sensitivity and specificity depending upon the particular panel of sera used for evaluation. Certain viral lysates may express one or another HIV epitope, such as p24 or gp42, more or less strongly. Also, individuals vary in their particular reactivities to these antigens and in the relative pattern of antibody expression. Therefore the pattern of banding on immunoblots may vary and criteria for positivity must be determined with care (Zuck, 1989).

MARKETING AND REGULATORY ISSUES

THE BIOTECHNOLOGY INDUSTRY

The production and marketing of immunodiagnostic reagents, even those produced by conventional methods, must be viewed in the context of the biotechnology industry. In some ways biotechnology is different from other businesses. There are often close and complicated links among governments, universities, and industry, of which two kinds are involved. The first is the big established transnational corporations, many of which already have branches in most countries of Latin America. The second, particularly in the United States, is the multitude of derivative specialized biotechnology companies, some with one or a few products, some with no products, but salable knowledge of some methodology or process. Many such companies have been set up by university scientists with funding from venture capitalists and/or larger established companies in the pharmaceutical or other industries. In developing countries the issues of organization, governance, and marketing policy are often more complex: "Weakness at the entrepreneurial level and the complexity of the interests tied to biotechnologies are reflected in the difficulty of defining a strategy for the sector" (Sorj and Wilkinson, 1988).

One of the most difficult problems facing the industry is that of property rights. The U.S. Patent Office has thousands of biotechnology applications from around the world sitting in boxes because there are

few people available to read them and to make decisions. Resultant delays of several years (or more) retard the transfer of technology because a company may not want to release or even to reveal a potentially useful method before it has secured patent protection. Ever then, the relative uniformity of R&D processes and resulting products has caused 1) many patents, if awarded, to be contested by other companies and 2) many lawsuits by patent holders against other companies, claiming infringement of patents.

There are two separate but interrelated issues: 1) R&D work in industrial countries on products intended specifically for the developing countries, and 2) Access to technology by the developing countries: through links between developing country institutions and industrial country partners, or by agreements for testing, production and/or marketing of products in their countries. Access may be limited by several mechanisms: trade secrecy, patents, or exclusive licensing agreements. Privatization may lead to lowered access to the technology and stimulate work on products with potentially large profit bases in the US and other industrial countries.

ADOPTING IMMUNODIAGNOSTIC PROCEDURES

A decision regarding the adoption of any immunodiagnostic procedure must consider not only its epidemiologic and economic rationale, but also the continuing and reliable availability of all needed components, including:

1. The primary test reagents, individually or as a kit;
2. Other enabling materials, such as
   a. Ancillary chemicals (e.g., buffers, gel consituents, visualizing enzymes and substrates);

b. Small supplies (microtiter plates, pipette tips, ...);

3. Needed equipment, properly calibrated (power supplies, water baths, freezers, incubators, fluorescent microscopes);
4. Adequate infrastructure such as transport and communications facilities, laboratory space, and electrical power;
5. Human resources: properly trained technicians and supervisors;
6. The ability to deliver sufficient numbers of test specimens to the laboratory in suitable condition;
7. The capacity to follow up on the findings of the procedure, for example, by providing treatment, or instituting public health measures.

Product life cycle. In addition, some forecast must be made about the useful life of the procedure within the national or local context. The advance of science never stops and a large investment in a particular technology may be jeopardized at any moment by a new discovery or superior method discovered elsewhere. One major immunodignostics manufacturer (personal communication) estimates the useful product life cycle in this field at 3 to 5 years, presenting risks that are better assumed by the big transnational corporations. The consequences of a commitment to a particular methodology or proprietary kit should therefore be evaluated with care. On the other hand, decisions cannot be deferred indefinitely while waiting for a perfect and permanent solution to a diagnostic dilemma.

IMPORTATION VS. LOCAL MANUFACTURE

Sourcing alternatives for pharmaceuticals and biologicals in developing countries include:

1. Importation of the finished product, packaged for final use;
2. Bulk importation of the finished product, with local packaging;
3. Importation of a product intermediary or precursor, with a local finishing process involving a chemical change, followed by local packaging;
4. Importation of raw materials with local production, finishing and packaging;
5. Local production from raw material to finished product, but using some imported supplies, technology, machinery and equipment;
6. Complete, self-sufficient, independent local production.

With particular reference to immunodiagnostic reagents, practical options are limited essentially to items 1, 2, and 5 in the list above. The reasons for selection of one or another of these alternatives for any product are complex, and include:

a. Size of the market: population base, usual prevalence of the condition, consumer preference;
b. Cost;
c. Sudden or unexpected need, as with an outbreak, epidemic, or appearance of a condition unusual for the country;
d. Local expertise in R&D and production;

e. Local laws and regulations, such as environmental controls, need for containment facilities for work with pathogenic organisms, or the like where applicable;

f. Availability of hard-currency foreign exchange;

g. Availability of internal resources, including distribution networks and appropriate laboratories;

h. Government policies, including bulk purchase by health ministries and importation duties and regulations;

i. Laws and regulations regarding patents, the transfer of technology, and local ownership of business enterprises.

For more common conditions, local manufacture may result in cost savings provided that the product volume and quality are high enough. Cost considerations include not only the price of the product itself but also all of the other resource elements necessary to reach a diagnostic conclusion based upon use of that product. Adaptations to the local situation can produce epidemiologically and economically effective products that support the public health goals of the nation.

On the other hand, importation is the only option for costly and complex products to be used in small quantities.

INTRAREGIONAL EXCHANGES OF MATERIALS

There is at least the theoretical possibility of interchange of immunodiagnostic reagents among countries of the region, more or less in barter arrangements that could spare the expenditure of foreign exchange. The exchange of small amounts of materials, e.g., among individual investigators and laboratories, has been in place for some time on an inform l basis. The interchange of moderate volumes of reagents for actual diagnostic use has been discussed in several

meetings sponsored by the Pan American Health Organization, and several difficulties have been identified. First, the scientific research and development capacity within the region is highly uneven. For example, one country offers conventional or even obsolete products having little present demand (such as Casoni skin-test antigens for echinococcosis). At the same time another country makes available 20 different monoclonal antibodies for discrimination of different kinds of tumor markers, which few laboratories in the region have the capacity to employ effectively. In both cases, the products find little application because they are aimed at an inappropriate technological level, either too low or too high. Second, the large transnational corporations have in many cases identified the needs, targeted the laboratory market, and provided suitable products with the aid of their substantial resources in market research and product development, and the personnel operating diagnostic laboratories prefer to work with known products. In some larger countries, such as Argentina, Brazil, and Mexico, domestic companies or local affiliates of transnational corporations may play a significant role.

THE MARKET FOR IMMUNODIAGNOSTIC REAGENTS

The worldwide market for all in vitro diagnostic products has been estimated at US$7.164 billion (B) for 1987, rising to $11B by 1990 and $13B by 1995. This includes products for clinical chemistry, hematology, drug monitoring, cancer testing, and other purposes. In vitro diagnostics for infectious diseases were estimated at 15% of the total market and diagnostics for blood processing at about 2% (Anon. 1988c). For immunoassay reagents, kits and instruments the 1990 wordwide market is estimated at $1.35B (Anon. 1988b). The United

States, with about 5% of the world population, accounts for about 40% of the total diagnostics market (Anon 1988a). Compiled data specifically for Latin America are generally unavailable.

The number of companies manufacturing diagnostic kits and products (including instrumentation) has been estimated at well over 1,000, of which about 300 are in the United States. However, about 50% of the total world market is held by five companies (Abbott, DuPont, Boehringer Mannheim, SmithKline Beckman, and Merck), and 75% is held by 15 companies (Charlish, 1988). Products for detection of HIV infection are made by more than 50 companies and represent a rapidly growing area, with nearly 40 million tests performed in 1987 (Anon. 1988d).

The pharmaceutical industry as a whole is robust in many areas of Latin America. Table 5 shows recent data for two countries.

DIAGNOSTICS IN LATIN AMERICA

In many countries of Latin America health care is available on three levels: 1) private, fee-for-service physician care for the wealthier segment of the population; 2) one or more Social Security systems for specified workers and their dependents; and 3) a public-sector system operated by the Ministry of Health (MOH) that may include primary health care posts, local clinics, and regional hospitals, for less affluent elements of the population.

Although conditions vary greatly, in many countries the bulk of specialized patient-specific diagnostic testing is done within the private sector and the Social Security system. The MOH and the Social Security system may function independently and purchase different products for use under different guidelines and conditions. The public

Table 5

The Pharmaceutical Industry in Two Latin American Countries

Country	Market $US	Companies			Employees
		Total	National	Foreign	
Mexico	915[1]	288	217	71	46,000
Brazil	1,695[2]	475	434	41	56,800

[1] Private sector

[2] Total

Source: Anon. 1988 e, f

sector may be in charge of screening programs, including those for HIV, HBV, and other relevant pathogens in blood destined for MOH facilities. The Social Security and private systems may institute additional screening procedures for donated blood. The MOH is usually responsible for the diagnostic aspects of control programs for endemic problems such as malaria, toxoplasmosis, or sexually transmitted diseases, and must also utilize diagnostics in the investigation of outbreaks of yellow fever, louse-borne typhus, dengue or other infections. In general, such uses constitute a relatively small proportion of the total market for immunodiagnostics.

In many areas of Latin America small laboratories may be found, perhaps associated with pharmacies or private clinics, in smaller cities and towns. These laboratories may perform a limited repertoire of tests, primarily pregnancy testing, blood and urine chemistries, VDRL, and screening for malaria, Chagas' disease or other conditions of local significance. The number of procedures performed by such laboratories is relatively small and quality control may be suboptimal.

For reasons both of promotion of national industries, and conservation of foreign exchange, it may be expected that expenditure of government (MOH) funds be directed more for items produced nationally, while there may be fewer restrictions on purchases in the private sector. In some cases in less affluent countries the costs of certain surveys, epidemiological investigations and control programs are subsidized by external donors such as the World Health Organization (WHO), Pan American Health Organization (PAHO), or U.S. Agency for International Development (AID). Diagnostic reagents may be paid for by these agencies, or underwritten by the pharmaceutical industry in

connection with studies of drug or vaccine efficacy. In general, outside donors do not fund ongoing needs.

Cost factors. The cost of imported immunodiagnostic products is increased in some countries because of customs duties that may exceed the initial value of the product. Local industrial, economic and political issues determine the imposition of such duties. Other things being equal, the resulting price differential may favor the purchase o domestically produced rather than imported reagents or kits. However, as the cost of a patient-specific diagnostic procedure may be a relatively small part of his or her overall management, and as the consequences of misdiagnosis may be great, other considerations may override cost in the selection of the diagnostic product. These decisions are made, not by the patients involved, but by the physician or perhaps the laboratory director for private patient-specific procedures; and by the relevant authorities who oversee transfusion services in the MOH or Social Security systems. Their perception of the relative quality and reliability of available immunodiagnostic reagents will be an important factor in their decision.

SELF-CONTAINED KITS

Some self-contained diagnostic kits are available for use independent of any laboratory. Examples of such "dipstick technology" are sold over-the-counter in the United states and elsewhere for determination of pregnancy and ovulation, monitoring of blood glusose levels, occult fecal blood, urinary tract infection, and similar applications. Presuming that the diagnostic technology has been worked cut, the design and manufacture of kits for field-based diagnosis of

certain infectious diseases should be a straightforward matter of research and development. Such kits would be intended for use by persons within the health care system rather than by members of the public. The twin issues of cost and quality control apply as with any other product, perhaps more stringently. Special problems concerning their use in the field in developing countries must also be carefully considered; e.g., physical robustness to withstand suboptimal transportation and handling; useful shelf life under tropical ambient conditions; inclusion of built-in positive and negative controls or definite endpoints so that untrained users can regularly obtain a result of sufficient predictive value to make the test kit epidemiologically and economically useful.

Several kits designed for field use in developing countries have been described (e.g., Sanborn, 1981). Some, based upon a plastic envelope microbiology (PEM) technique, are intended for diagnosis of Candida albicans, Trichomonas vaginalis and Gardnerella vaginalis (Brady et al., 1986; Ching et al., 1988). In these kits all necessary reagents are enclosed within a plastic bag, requiring only the inoculation of a patient- derived specimen. Some PEM configurations have several reagents pre-packaged in sealed subunits, which can be broken open by pressure applied from outside the sealed pouch. Such units are designed to be entirely self-contained and usable in areas where no laboratories are available. The practicality of such products for field use remains to be determined.

QUALITY CONTROL OF BIOLOGICALS AND IMMUNODIAGNOSTIC REAGENTS

SPECIFICATIONS

Two elements are needed for complete quality control of immunodiagnostic procedures. The first is definition, specification and criteria for standardized reagents. The second is quality control, in the more conventional sense, of production and use of the products. This is discussed in detail below.

An example of product specification is the collaborative trials of laboratories organized by the WHO and IUIS (International Union of Immunologic Sciences) Subcommittee for Standardization to evaluate preparations of horseradish peroxidase-labelled sheep anti-human IgG in order to select a candidate with favorable properties for establishment of an international reference standard (Houba, 1981).

In the United States, standards for biological products including immunology and microbiology devices and reagents that are in commercial distribution are governed under the Code of Federal Regulations, Title 21, chapter 1, subchapter F, that covers the Food and Drug Administration. Section 610 describes general requirements of safety, potency, sterility, purity, and so forth. Additional and more specific requirements are set forth in section 660 and particularly in section 866 which includes microbiologic media and apparatus. Dozens of serological reagents for viral, bacterial and parasitic pathogens ranging from Acinetobacter calcoaceticus to Vibrio cholerae are specified in subpart D, immunology laboratory equipment and reagents in subpart E, and immunological test systems for blood factors and

components, and autoimmune diseases are listed in subpart F. These standards are widely recognized as a basis for regulation and as a focus for technical "harmonization activities" by other countries, by regional groupings such as the European Economic Community, and by advisory committees of international agencies such as the World Health Organization.

Specifications have been prepared in the United States for monitoring the safety, purity and potency of new drugs and biologicals produced by recombinant DNA technology (Liu, Gates and Goldman 1985). These are extensions of the U. S. government regulations normally applicable to biologics as described above. Data should be maintained on the consistency of yield of the product from full-scale culture, and criteria should be established a priori for the rejection of culture lots. Such products should not contain detectable viruses, nucleic acids or antigenic materials. As an example of the detal required, the following tests are specified (details omitted):

A. Physicochemical characterization of the proteins

1. Compositional analysis (amino acid composition)
2. Partial sequence analysis
3. Peptide mapping
4. Polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) to verify purity and molecular weight.
5. HPLC, especially reverse phase, to characterize and quantitate specific impurities.
6. Circular dichroism and optical rotatory dispersion (CD & ORD) to confirm conformational identity between recombinant DNA products and native reference materials.
7. Other characterizations, as appropriate.

B. Biological tests for identity and potency

The recombinant product should be compared with natural material in appropriate animal or in vitro bioassays.

C. Tests for contamination

1. Pyrogen contamination by Limulus amebocyte lysate, or animal testing
2. Viral contamination; by appropriate cell culture methods.
3. Nucleic acid contamination by observing the extent of removal of specifically added radiolabelled host cell DNA, plus hybridization tests
4. Antigen contamination. Western blots, radioimmunoassays (RIA), and enzyme-linked immunosprbent (ELISA) tests can be done, to detect contaminating antigens in a range of 1 to 100 ppm.
5. Microbial contamination. Appropriate microbiological tests should be performed to detect the presence of anaerobic and aerobic bacteria, fungi, yeasts and mycoplasma.

D. Toxicity tests in animals

QUALITY CONTROL AND TECHNICAL SUPPORT

Immunodiagnostic reagents must be produced with adherence to the principles of Good Manufacturing Practices (GMP) as defined in industry and governmental standards such as those listed above. Such testing requires a large investment in equipment, supplies, and personnel.

As biological products, immunodiagnostics may be affected by many conditions that can alter the sensitivity and specificity of tests in which they are employed. The history of cell culture work is strewn with stories of mysterious disasters, traced to changes in glass composition, dishwashing detergents, pipetting machines, personal

attributes of technicians, or similar esoteric factors. The quality and uniformity of raw materials is paramount. Plastic and glassware and culture media including salts, vitamins, amino acids, and other components must be absolutely consistent and must not leach components. Even so, the characteristics of monoclonal antibodies may change over time as hybridoma cells are gradually altered by slightly differing culture conditions or by changes inherent in the cells themselves. Hybridoma cells may suddenly stop producing antibodies, or produce at lower yields. For this reason, subcloned stocks of authentic hybridomas of known character should be maintained in liquid nitrogen and thawed as needed to maintain product quality, but it may take some time for such cells to secrete optimal amounts of antibody.

Water. A factor that causes perennial problems in culture systems is water. Reagents or powdered media dissolved in unreliable water can yield poor, inconsistent, or no product. Purification of water, e.g., by means of ion exchange, reverse osmosis and redistillation is necessary, but even if adequate and costly equipment is installed the water may be degraded over time by such factors as seasonal changes in raw water supply, exhaustion of resins, and accumulation of salts in the boiling chamber. Continual monitoring of water quality, is required, including regular maintenance of distillation equipment.

Serum. A second important element in hybridoma culture is serum. Natural fetal bovine serum (FBS) may be contaminated with viruses, mycoplasma or other organisms, or with hemoglobin, antibodies, interferon, complement, DNA and other components that can reduce prodictivity or damage cell lines [Note: bovine serum collected in South America can not be imported into the United States because of the

possibility of contamination with foot and mouth virus]. Sera vary greatly depending upon the type of cattle and location or season of collection, they are subject to large cost fluctuations, and their supply may be unpredictable. Each lot of FBS must be tested separately for growth-promoting properties with each hybridoma line. The use of available chemically defined commercial synthetic serum substitutes eliminates these problems, but generally at the expense of substantially lowered yields.

The standards of GMP, as well as government regulations, may be quite burdensome. For these reasons, experienced persons in the commercial immunodiagnostics industry recommend that for each individual engaged in manufacturing, there be one person for quality control, to run continuous checks on all aspects of the process, and to assure product compliance with established standards.

Technical support is required not only to backstop quality control personnel but also to serve as liaison between the R&D (research and development) and manufacturing arms of the operation. In this way, more cost-effective technological innovations can be incorporated into production and possibly into the utilization of the immunodiagnostic product.

Biohazards. Another aspect of technical support involves the protection of R&D and production workers, and of the surrounding environment, from potentially damaging organisms and procedures. Construction of P3 or other containment facilties may be needed to prevent the potential release of infectious waste materials, including pathogens and recombinant organisms. Such releases can pose a danger to the environment and to nearby populations, and can be harmful to the reputation of the company.

Biological relevance. Immunodiagnostic tests must reflect the biological and epidemiological realities in the region in which they are to be employed. New diseases such as HIV can appear unexpectedly. The relative frequency of different strains of pathogens may change over time. This is well known with influenza viruses but can occur also with other organisms. Immunodiagnostic reagents must be keyed to the particular organisms current in the area in which they are to be used. Tests that are too highly specific may fail to track genetic changes that occur in the natural history of many disease agents.

Scale of Production. Associated with quality control is the related issue of the scale of production. Given suitable materials and sufficient time, most diagnostic procedures can be carried out adequately on an investigational basis by a trained researcher and a few technicians. However, the reliable supply of production quantities of uniform diagnostics, on a cost-effective and profitable basis, is a different matter. One person in industry remarked that "It is one thing to make a few hundred coated beads for a research project, and quite another to produce a million identically coated beads every week. Scaling up is a major problem ".

Shelf life. The scale of production is linked closely to supply and demand: the supply of raw materials and demand for the product. This is particularly important in view of the relatively short shelf life for many immunodiagnostic products, and the usual requirement for their storage under refrigeration, or by freezing at -20C or even -70C. Household-type -20C freezers of the "self-defrosting" type are unsuitable for storage of immunoreagents because the repeated cycles of freezing and warming are known to damage the delicate antisera.

Product life cycle. As described previously, an important element in product life is the useful period of time during which the particular immunodiagnostic product is technologically competitive. The many companies in the international diagnostics marketplace spend millions of dollars annually for research and development of new products, and also adapt published academic research findings. In this technological environment the useful life span of a particular immunodiagnostic product may be only a few years, during which investments in R&D and specialized production requisites must be recovered through economies of scale.

QUALITY ASSURANCE IN PRODUCT UTILIZATION

The same issues of economy and quality confront the users as well as the manufacturers of immunodiagnostics. Many recent manuals emphasize the magnitude of quality issues in the clinical laboratory. For example:

> ... quality assurance includes all actions necessary to provide adequate confidence that test results satisfy given requirements or standards. Laboratory quality assurance should thus be thought of in much broader terms, as a system to prevent or control errors that occur from the time a test is ordered until the time it is interpreted ... Surveillance of laboratory procedures should be systematic, periodic, and documented, but quality assurance is an expensive, time-consuming task that must be balanced with prompt, efficient service. Laboratory quality control procedures generate 30 percent or more of a laboratory's costs. (Howanitz and Howanitz, 1987)

Lapses in technique will cause even the best reagents to perform below their design potential. Many investigators have followed the performance of different laboratories on aliquots of a single test specimen. The results are often not encouraging. For example, Kagan (1982) showed the data obtained by six laboratories (presumably in the United States) which all performed a Sabin-Feldman dye test for

toxoplasmosis on batches of the same antiserum. Dye test titers ranged from 1:1250 to 1:10,000; determination of IgM varied 16-fold and IgG titer from 160 in one laboratory to 12,800 in another. There are clearly an infinite number of ways to go wrong, but only a single route to perfection. The range of factors that can lead to degraded performance is beyond the present discussion, but some are shown on Table 6.

Technical proficiency of laboratories should be determined routinely by use of coded specimens of known diagnosis, and should be built into any plan for production or distribution of immunodiagnostic reagents. Such performance must be evaluated before any large-scale seroepidemiological study is contemplated, and from time to time during the course of the project.

The geographic dispersion or concentration of laboratory facilities in any country is a reflection of population distribution, economics, and governmental policies regarding need, access, equity, and other service-related factors. The marginal costs of diagnostic tests are lower and their accuracy (predictive value) is greater when they are performed regularly in larger centralized laboratories rather than occasionally in local facilities, provided that means are available to transport the specimen in good condition to the laboratory. Total costs must be viewed in terms of the total resource expenditure for facilities, equipment, supplies, personnel (including training), and quality control. As mentioned above, there is no reasonable alternative to spending money for quality control, which is an essential component of the laboratory testing program.

Table 6

Requisites for Quality Assurance
for Immunodiagnostic Procedures

Adequate preliminary research and development of the product

Adherence to Good Manufacturing Practices

Proper packaging, shipping, storage, and use before the expiration date

Suitably trained and motivated personnel

Supportive administrative and institutional framework

Refresher and in-service courses to maintain and update skills

Proper collection, preservation, shipping and handling of specimens

Use of appropriate methods

Suitable design, construction, and maintenance of laboratory space

Testing of components such as media, stains and reagents

Maintaining equipment in good repair and proper calibration

Using appropriate controls and standards

Maintaining proper records

Proficiency testing of personnel by coded control specimens

Use of split or duplicate samples fo consistency testing

Comparing results with other laboratories

Communication with the persons who sent the specimens and with those who will act on the diagnosis

Acting to correct lapses and departures from standards or quality

APPLICATIONS OF IMMUNODIAGNOSIS TO PRIMARY HEALTH CARE

The worldwide movement toward primary health care (PHC) received its impetus at the International Conference on Primary Health Care held at Alma Ata, USSR, in 1978. According to the Declaration issued at that conference, PHC was defined as:

> ...essential health care based on practical, scientifically sound and socially acceptable methods and technology made universally accessible to individuals and families in the community through their full participation and at a cost that the community and country can afford to maintain at every stage of their development in a spirit of self-reliance and self-determination.

Many communities in the region are medically underserved to the extent that even primary health care is difficult to achieve. In an era of financial stringency brought about by the international debt crisis, with 20 billion dollars a year flowing from the poor countries to the rich countries, and with pressure by international agencies for more austerity, there is little surplus in many areas for nonessential procedures. In fact, there is often not enough for essential activities. Is it not, therefore, a contradiction to consider the role of clinical laboratories for primary health care in places where many of the entries on table 1 have little or no reality? Is there any role here for a diagnostic laboratory or for immunodiagnostic reagents?

The most likely answer is: that depends the purpose of the procedure. For management of childhood diarrheal disease, there is not much need to specificy the etiologic agents because in most cases the

recommended treatment is the same: oral rehydration therapy. However, there are circumstances in which an accurate diagnosis is critical. The great epidemic of dysentery that struck parts of Central America in the 1960s and 1970s was assumed to be amebiasis due to E. histolytica before the laboratory showed the agent to be Shigella dysenteriae type I, which is an entirely different matter.

In an isolated rural community there is no realistic way that such a diagnosis can be made locally. The major diagnostic tool at the village level is the interest and observational skill of the local health worker, and it is likely to remain so at least for some years. It is a truism that common things are commonly observed. In an area endemic for malaria, schistosomiasis or Chagas' disease, the astute health worker can recognize and treat these with a high degree of accuracy and, with minimal laboratory services.

A significant step in community diagnosis can be the support of "primary epidemiologic monitoring". Village health workers, parteras or curanderos, or even the school teachers, can be alerted to be aware of unusual clusters or outbreaks of illnesses and to report these to local health authorities for diagnostic workup.

According to a common plan, laboratories in developing countries are characterized as peripheral, intermediate, or central. At what level of the health care system should the first laboratory be found? Rather than establish many poorly equipped and dysfuntional small laboratories operated by the government in rural or poor urban areas, efforts should be made to teach primary health workers how to obtain and safeguard appropriate clinical specimens, and how to send or transport these to the nearest competent laboratory.

Imperato (1985) has written:

> The rational distribution of funds for laboratory equipment an personnel between the large urban centers and rural areas is frequently jeopardized in the budgetary process in less developed countries by political imperatives that tend to shift most of the resources to the urban centers where most of the higher skilled physicians practice and where unmet needs and wants of the population can rapidly produce political consequences. Within health budgets in less developed countries as in developed countries, diagnostics must compete with therapeutic costs which are often extremely high since most drugs must be imported and more importantly with personnel services costs which in many countries often reaches seventy percent of a total health budget.

It seems reasonable and necessary that essential diagnostic capabilities be incorporated, according to recommended WHO standards, at the first level of the health care system that has a physician in attendance, commonly a district hospital or larger health center. This laboratory should perform basic chemistry, hematology, and the like, pregnancy testing, and tests for major vectorborne, parasitic, and infectious (including sexually transmitted) diseases, including many shown on Table 1. In addition to its clinical routine, such a laboratory may function within the public health system in epidemiologic study or ascertainment or in limited environmental testing (e.g., of water). The functioning of this laboratory at an acceptable level demands attention to quality control, which means a plan for continuous monitoring. The usual hierarchy of levels of referral would mandate increasingly competent laboratories through intermediate (regional and provincial) levels to the national referenc center.

The twin issues of cost and quality are foremost in primary health care, to help determine which diagnostic services are provided, and materials purchased, by governments or sometimes by external donors. Because funds are always limited, costs must be reduced to a minimum.

Because of the large fixed investments in facilities, equipment and salaries, economies of scale would argue for larger laboratories with lower unit costs, but this is exactly contrary to the intention of providing diagnostic services for primary health care at the local level. An efficient transport system, as mentioned, can help.

The issue of cost is intimately associated with quality, as a misleading or erroneous test is no bargain even if it is free.

Quality control is a way of life both in the manufacturing of reagents and in the performance of the appropriate test. The weakest link will set the standard for the value of the entire process. The difficulties of maintaining adequate standards in laboratories remote from large centers are apparent from a study of Table 5.

Every diagnostic method must be evaluated and justified solely to the extent that the efficiency and effectiveness of health services are improved by its adoption. Every procedure now considered standard, including electric lights and running water, was at one time an innovation. Therefore novelty alone should not stand for or against the adoption of a diagnostic method, only its predictive value and cost effectiveness.

Innovations are very situational in place and time. Many new procedures, well established in one setting, fail to survive the hazards of technology transfer. A costly diagnostic instrument may be useless in an inappropriate setting through lack of reliable electric power, or trained personnel, or spare parts, or maintenance, or because nobody can afford to use it, or because even if it works perfectly the condition that is diagnosed can't be treated anyway, or maybe because the disease that it is designed to uncover doesn't occur there.

Technologic innovation in industry, with good manufacturing practices and economies of scale, can produce diagnostic products with excellent sensitivity and specificity, in large volume, and inexpensively on a unit basis. However, their utilization may make patient care more costly and complex. First, there must be decisions about which products to use, less familiarity with them, and greater uncertainty and chance for error. Second, tests may be more sensitive and specific, can find more things and find them earlier and therefore demand additional services. Many minor conditions that would normally disappear by themselves would now demand some response from the medical care system. Therefore, while innovation may lead to better, more effective, more diverse and cheaper diagnostic products, the total resource cost involved in their utilization may be greater.

The fact that a product or procedure is functionally sound is a necessary but not a sufficient condition for its adoption. Referring specifically to new diagnostic techniques, Nelson (1986) has pointed out that 60,000 people in India who make a living by reading malaria slides would be displaced by adoption of novel methodologies for malaria detection. Can there then be some socially compelling reason not to adopt an innovation?

New products made by methods of biotechnology have been, or will be, proposed for the diagnosis of many diseases in developing countries. But it must be proven that they are logistically simpler, more cost-effective, more acceptable, be more sustainable and are generally superior to existing methods, if any, or to other appropriate routes to accomplishing the same end.

This is especially true of derivative or technology-driven

innovation, for example, the use of molecular cloning, PCR, DNA hybridization, or hybridoma production. Once the methodologic cascade is defined, as in the case of producing a monoclonal antibody, the competitive commercial environment can generate a series of products that somebody thinks may be useful in primary health care in underserved areas. But as mentioned, evaluation in the field is difficult. Can busy local health workers be expected to spend time on this activity without direct subsidization, and will this testing of tests take time away from the basic service work of laboratory personnel?

What is needed is a method for preliminary screening, by computer simulation, epidemiologic and econometric modeling, or similar means, to select those competing products or methods with the greatest likelihood of success in a real field trial.

Nevertheless, there are certain benefits to implementing advanced technologies for diagnosis, or other purposes, in developing countries. Science is a worldwide activity and all countries should be involved. Technology transfer can help to reduce the emigration of scientists and technicians, at the same time stimulating research and education at a national level. In a more practical sense, where there is a condition of only local significance, imported diagnostic products are likely to be unavailable and national competence in the basic R&D and manufacturing technology can produce an appropriate reagent.

Caution is always necessary. Some diagnostic technologies, such as radiolabelled DNA hybridization probes or methods that use toxic materials, may present environmental problems in use and disposal.

The possible development and deployment of self-contained diagnostic kits has been discussed earlier. The development and distribution of such "dipstick technology" for use in primary health care posts could be a significant advance in extending the capablities of community health care workers.

SUMMARY AND RECOMMENDATIONS

Diagnostic testing and screening can contribute to better health through prevention and treatment of individuals and disease control in communities. All countries are now faced with the challenge of providing adequate diagnoscic services despite limited financial, human, and laboratory resources.

Current technologies

Among the immundiagnostic procedures available, the classical methods of agglutination, complement fixation, precipitation, and immunofluorescence remain useful and popular. More modern enzyme immunoassay procedures such as ELISA, immunoblotting, and immunocytochemical techniques have become widely available in recent years. These depend upon the attachment of an enzyme to a known antigen (or antibody), which is then incubated with the test specimen. Presence of the sought-for antibody (or antigen) will result in immunological binding, which will include the attached enzyme. A further incubation with an appropriate substrate will produce a colored product that will indicate the concentration (in ELISA and blotting) or location (in immunocytochemistry) of the reactive element in the test specimen. The development of monoclonal antibodies in the mid-1970s and their current wide availability offers a highly sensitive and specific method for identification of antigens, including those of pathogenic organisms as well as malignant or other abnormal cells.

Although non-immunologic, nucleic acid hybridization technology is rapidly gaining in importance, particularly since the development of the polymerase chain reaction and other means of amplification of specific nucleic acid sequences. These powerful tools are moving out of the research laboratory and will become a prominent part of the diagnostic scene within the next few years.

Screening of blood

The screening of blood for transfusion has become an important issue in all countries. Among the pathogens that may be transmitted are the human immunodeficiency, hepatitis B and certain other viruses, Trypanosoma cruzi, Toxoplasma gondii, and Plasmodium species.

Local manufacture versus importation

This issue is complicated by the imprecise meaning of "local manufacture." At one extreme the term may indicate the invention, research, development and manufacture of an indigenous product by a locally-owned entity; at the other, it may refer to the finishing or packaging of an imported bulk precursor within a local plant of a large transnational corporation. Recognizing this ambiguity, and the great variation among so-called developing countries, it is possible only to state general principles.

Potential benefits of local manufacture:

1. Capability-building of local industry, including training of personnel; reduction of technological dependence;
2. Economic benefits such as saving of foreign exchange, retention of profits locally, tax advantages, avoidance of import duties, and possibly lower product cost;
3. Ability to tailor product to local needs and use imported technology more effectively;

4. Positive interaction with local government and universities;
5. Reduction in emigration, or repatriation or attraction of professionals.

Potential risks of local manufacture:

1. Limitations in the local market (caused by population size, prevalence of the condition, expendable funds, professional or consumer preference) may lead to insufficient volume or turnover for products with a limited shelf life;
2. Probable continued dependence on imported components, materials or equipment for some portion of the production process, with expenditures of foreign exchange;
3. Probability of product obsolescence unless there is a robust and continuing research and development program;
4. Competitive and economic pressures from larger suppliers;
5. Possible patent or regulatory issues;
6. Relatively small base of expertise for technical support such as solving production or quality control issues.
7. Exposure to liability in the event of poor product performance

Implementation of diagnostic procedures

The implementation of any diagnostic method depends on:

1. The public health significance of the pathogen or disease;
2. Continuing availability of the test reagents;
3. Presence and reliability of enabling infrastructure- product distribution networks, laboratories with trained technicians, reagents, apparatus and equipment, communications;
4. Cost of the procedure in relation to- patient's ability and willingness to pay in the case of fee-for-service practice; sustainable budgets of third party payers such as the Seguro Social or Ministry of Health;

5. Predictive value of the test for the particular disease or condition;
6. The capacity to follow up on the findings, e.g., by providing treatment or instituting public health measures;
7. Knowledge and interest in the procedure by the public, the medical profession and/or the public health authorities.

Recommendations

The deployment of immunodiagnostic products in any country must be in harmony with national health policies, and should be undertaken only where conditions for implementation (listed above) are favorable. For newly introduced products it must be proven that they are logistically simpler, more effective, more acceptable, be more sustainable, and are generally superior to existing methods, if any, or to other appropriate routes to accomplishing the same diagnostic ends.

For diagnostic and screening procedures for diseases of public health significance, for which public funds must be expended, decisions about the local production of reagents must be based on issues of cost and quality. In larger countries of the region, a well-developed biotechnology industry includes local companies with more or less affiliation with transnational corporations, as well as direct subsidiaries. Here there is likely to be sufficient expertise for production of quality- controlled reagents, and economic issues such as market volume and the supply of investment capital or foreign exchange will contribute to decisions about local production. In smaller countries with an insufficient scientific and technologic base, scale-up to reliable, commercially viable production of a reagent of uniform quality appears less feasible, and is probably not justified.

REFERENCES

Anon. 1988a. Diagnostic market analysis. Clinica 270:16

Anon. 1988b. Diagnostic market set for new growth. Clinica 283:17.

Anon. 1988c. 40 million HIV tests in US in 1987.

Anon. 1988d. World IVD market analysis. Clinica 314:14.

Anon. 1988e. The Mexican pharmaceutical industry- an overview. Scrip 1359:20--22.

Anon. 1988f. Brazilian '87 pharma sales. Scrip 1369:20.

Basch PF. 1989. A Textbook of International Health. New York. Oxford University Press. Forthcoming.

Berner JJ. 1984. Effects of Diseases on Laboratory Tests. Philadephia. JB Lippincott.

Brady WK, Paine DD, Frye LP, Sarno AP, Purdon A Jr, Borchardt KA, Engelkirk PG. 1986. Evaluation of new plastic envelope microbiology (PEM) methods as adjuncts in the diagnosis of Candida albicans and Trichomonas vaginalis vaginitis, Military Med 151:478-480

Burnett JT. 1983. Textbook of Immunology. 4th Edition. St. Louis, CV Mosby.

Charlish P. 1988. The European in vitro diagnostics industry in perspective. Clinica 304:15-18.

Ching LQ, Borchardt KA, Smith RF, Beal CB. 1988. A 24 hour plastic envelope method for isolating and identifying Gardnerella vaginalis (PEM-GVA). Genitourin Med 64:180-184.

de Shazo RD, Lopez M, Salvaggio JE. 1987. Use and interpretation of diagnostic immunologic laboratory tests. J Amer Med Ass 258:3011-3031.

Dodd RY. 1988. Research in progress workshop. Transfusion Medicine Reviews 2:299-300.

Draper CC, McLaren ML. 1981. Some applications of immunoassays in tropical parasitic infections. In A Voller, A Bartless, D Bidwell, Eds. Immunology for the 1980s. Baltimore. University Park Press. p. 449-456. Greenwood BM, Whittle HC. 1981.Immunology of Medicine in the Tropics. London. Edward Arnold.

Duggan DB, Ehrlich GD, Davey FP, Kwok S, Sninsky J, Goldberg J, Baltrucki L, Poiesz BJ. 1988. HTLV-1-induced lymphoma mimicking Hodgkin's Disease. Diagnosis by polymerase chain reaction amplification of specific HTLV-1 sequences in tumor DNA. Blood 71:1027-1032.

Elwing H, Nilsson L-A. 1980. Diffusion-in-gel immunoassay (DIG-TIA): optimal conditions for qualtitation of antibodies. J Immonol Meth. 38:257-268.

Elwing H, Nilsson L-A, Ouchterlony O. 1977. A simple spot technique for thin layer immunoassays (TIA) on plastic surfaces. J Immunol Meth. 13:131-145.

Elwing H, Nygren H. 1979. Diffusion in gel-enzyme-linked immunosorbent assay (DIG-ELISA): a simple method for quantitation of class-specific antibodies. J Immunol Meth 31:101-107.

Friedman RB, Anderson RE, Entine SM, Hirshberg SB. 1980. Effects of diseases on clinical laboratory tests. Clin Chem. 26:1D-476D.

Ganju L, Gaur A, Talwar GP. 1988. Use of filter paper discs as substrate for collection and storage of blood samples for screening of anti-tetanus toxoid antibodies. Diagn and Clin Immunol 5:262-265.

Giaever I. 1973. The antibody-antigen reaction: a visual observation. J Immunol 110:1424-1426.

Greenwood BM, Whittle HC. 1981. Immunodiagnosis of infectious diseases In BM Greenwood and HC Whittle. Immunology of Medicinein the Tropics. London. Edward Arnold. p. 136-177.

Grieco MH, Meriney DK. 1983. Immunodiagnosis for Clinicians: Interpretation of Immunoassays. Chicago. Yearbook Medical publishers.

Guatelli JC, Gingeras TR, Richman DD. 1989. Nucleic acid amplification in vitro: detection of sequences with low copy numbers and application to diagnosis of human immunodeficiency virus type 1 infection. Clin Microbiol Reviews 2:217-226.

Houba V. 1981. The enzyme-linked immunosorbent assay (ELISA). In RH Morrow, Ed. Report of an Informal Consultation on Further Development of Diagnostic Methods for Tropical Diseases. Geneva. UNDP/World Bank/WHO Special Programme for Research and Training in Trpoical Diseases. p. 67-71.

Howanitz PJ, Howanitz JH. 1987. Laboratory Quality Assurance. New York. Mc-Graw Hill Book Company.

Imperato PJ. 1985. The potential of diagnostics for improving community health in less developed countries. J Community Hlth. 10:201-206.

IUIS/WHO Working Group. 1988. Laboratory investigationsin clinical immunology: methods, pitfalls, and clinical indications. Clin Immunol Immunopathol, 49:478-497.

Kagan, I.G. 1962. Standardization and quality control of immunodiagnostic methods. Behring Institut Mitteilungen 71:10-22.

Kaçan ID. 1986. Serodiagnosis of parasitic diseases. In NR Rose, H Friedman, JL Fahey, Eds. Manual of Clinical Laboratory Immunology. 3d Edition. Washington, DC. American Society for Microbiology. p. 467-487.

Kingsbury DT. DNA probes in the diagnosis of genetic and infectious diseases. Trends in Biotechnology 5:107-111.

Klausner A. 1987. Visible immunodiagnostics vie with ELISA. Bio/Technology 5:8-9.

Kühnl P, Seidl S, Böhn BO. 1989. Reduction of virus load in blood donations by screening methods. Current Studies in Hematology and Blood Transfusion 56:9-22.

Kurstak E. 1985. Progress in enzyme immunoassays: production of reagents, experimental design, and interpretation. Bulletin of the WHO 63: 793-811.

Landry ML, Fong CKY. 1985. Nucleic acid hybridization in the diagnosis of viral infections. Clinics in Lab Med 45:524-529.

Larsen J, Odell WD. 1986. General principles of radioimmunoassay. In NR Rose, H Friedman, JL Fahey, Eds. Manual of Clinical Laboratory Immunology. 3d Edition. Washington, DC. American Society for Microbiology. p. 110-115.

Liu DT, Gates FT III, Goldman ND. 1985. Quality control of biologicals produced by rDNA technology. Devel Biol Standard 59:161-166.

McDade JE. 1985. Diagnostic applications of monoclonal antibodies: infectious disease diagnosis. In DS Gordon Ed. Monoclonal Antibodies in Clinical Diagnostic Medicine. New York. Igaku-Shoin. p. 137-158.

Maggio ET. 1982. Advances in enzyme immunoassay: methods and applications. In RM Nakamura, WR Dito, ES Tucker, Eds. Clinical Laboratory Assays: New Technology and Future Directions. New York. Masson Publishing. p/ 17-31.

Nakamura RM, Robbins BA. 1986. Analytical fluid-phase fluorescene immunoassays. In NR Rose, H Friedman, JL Fahey, Eds. Manual of Clinical Laboratory Immunology. 3d Edition. Washington, DC. American Society for Microbiology. p. 116-123.

Nelson G. 1986. Opening remarks. Parasitology 92 (Suppl.):S3-S5.

Nichols WS, Nakamura RM. 1986. Agglutination and agglutination inhibition assays. In NR Rose, H Friedman, JL Fahey, Eds. Manual of Clinical Laboratory Immunology. 3d Edition. Washington, DC. American Society for Microbiology. p. 49-56.

Nilsson L-A, Björck L, Ouchterlony Ö. 1985. Paper discs impregnated with capillary blood. A sampling technique for immunoassays by means of DIG-ELISA and DIG-TIA. Trans Roy Soc Trop Med Hyg. 79:314-318.

Palmer DF, Whaley SD. 1986. Complement fixation test. In NR Rose, H Friedman, JL Fahey, Eds. Manual of Clinical Laboratory Immunology. 3d Edition. Washington, DC. American Society for Microbiology. p. 57-66.

Payne WJ, Marshall DL, Shockley RK, Martin WJ. 1988. Clinical laboratory applications of monoclonal antibodies. Clin Microbiol Reviews 1:313-329.

Pollice M, Yang H-L. 1985. Use of nonradioative DNA probes for the detection of infectious bacteria. Clinics in Lab Med 5:463-473.

Sanborn WR. Use in tropical areas of a portable kit for rapid diagnosis of infectious diseases. WHO Chronicle 35:54-56.

Sauls CD, Caskey CT. Applications of recombinant DNA to pathological diagnosis. Clin Chem 31:804-811.

Seeff LB. 1988. Transfusion-associated hepatitis B: past and present. Transfusion Medicine Reviews 2:204-214.

Sorj B, Wilkinson J. 1988. Brazilian policies in biotechnologies: a post-ethanol strategy? Bio/Technology 6:150;154-155.

Soulier JP, Prou O, Dubarry M. 1985. Plasmodium falciparum antigen detection with monoclonal antibodies. Develop in Biol Standard 62:21-27.

Tam MR, Goldstein LC, Yelton DE. 1985. Monoclonal antibodies in clinical microbiology. In EH Lennette, A Balows, WJ Hauser, HJ Shadomy, Eds. Manual of Clinical Microbiology. Washington. American Society fo Microbiology. p. 905-909

Van Brunt J. 1988. HIV screening: matching technology to markets. Bio/Technology 6:259-264, passim.

Van de Perre P, Nzaramba D, Allen S, Riggin CH, Sprecher-Goldberger S, Butzler J-P. 1988. Comparison of six serological assays for human immunodeficiency virus antibody detection in developing countries. J Clin Microbiol. 26:552-556.

Voller A, 1985. Serodiagnosis of tropical parasitic diseases with special reference to the standardization of labelled reagent tests. Develop. Biol. Standard. 62:3-9.

Voller A, Bidwell D. 1986. Enzyme-linked immunosorbent assay. In NR Rose, H Friedman, JL Fahey, Eds. Manual of Clinical Laboratory Immunology. 3d Edition. Washington, DC. American Society for Microbiology. p. 99-109.

Voller A, De Savigny D. 1981. Diagnostic serology of tropical parasitic diseases. J Immunol Meth. 46:1-29.

WHO. (no date). Programme for Vaccine Development (brochure). Geneva. World Health Organization.

Zuck TF. 1989. How should we test the tests? Transfusion 29:3-4.