- MEASURING STRESS

A Guide for Health and Social

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10

Measurement of Immune Response

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Preliminary Considerations

The field of psychoneuroimmunology (PNI) has grown very rapidly in the last decade; a number of studies have shown immunological alterations in response to commonplace stressful events such as academic examinations (Glaser et al., 1990), as well as transient laboratory stressors such as mental arithmetic (Kiecolt-Glaser, Cacioppo, Malarkey, & Glaser, 1992). In addition, although data are limited, chronic stressors have been linked to the longer-term down-regulation of immune function (Baum, Cohen, & Hall, 1993; Kiecolt-Glaser, Dura, Speicher, Trask, & Glaser, 1991), and diverse interventions appear capable of modulating various aspects of immune function (Kiecolt-Glaser & Glaser, 1992). Although the evidence is still preliminary, these immunological changes appear to have consequences for health (Cohen, Tyrrell, & Smith, 1991; Glaser et al., 1987; Kasl, Evans, & Niederman, 1979).

In the first part of this chapter, we provide a very basic overview of some of the important concepts and terms (see also Table 10.1). For more detailed information on behavioral influences on immune function, the reader can consult several sources (Ader, Felten, & Cohen, 1991; Andersen, Kiecolt-Glaser, & Glaser, 1994; Glaser& Kiecolt-Glaser, 1994x, 1994b; Herbert & Cohen, 1993x, 1993b; Kiecolt-Glaser & Glaser, 1992). For further discussions of basic immunology, the November 25, 1992 issue of JAMA (Volume 268) provides a primer on allergic and immunological diseases, with 26 articles covering clinically relevant immunology; in addition, there are a number of immunology textbooks (e.g., Male, Champion, Cooke. & Owen, 1991; Stites & Terr, 1991).

Basic Immunological Terms and Concepts

Table 10.1. Glossary of Important Concepts and Terms

Antibodies	Inununoglobulins. In humans, there are five major classes of antibodies-IgG, IgA, 1gM, IgE, and IgD. Some antibodies can neutralize the effects of toxins; others can lyre cell membranes; whereas the IgE class is involved in hypersensitivity reactions.
Blastogenesis	Term used to describe the induction of cell division after exposure to a mitogen or an antigen. Blastogenesis is thought to provide an in vitro model of the lymphocyte proliferatioe response to challenge by infectious agents.
B-lymphocytes	Lymphocytes that produce immunoglobulins, and the primary cells associated with the humoral immune system; derived from bone marrow.
Cellular immune response	Immune functions not involving antibody but involving primarily T-lymphocytes. Cellular immunity is particularly important for the defense against intracellular viruses, transplanted tissue, cancer cells, fungi, and protozoans.
Helper T -lymphocytes	Cells that stimulate the production of immunoglobulins by B-lymphocytes.
Humorai immune of response	The arm of the immune response responsible for the production immunoglobins.
Hypersensitivity	Allergy, or the enhanced responsiveness of the immune system to a foreign substance which leads to pathological tissue changes. Immediate hypersensitivity reactions can occur in minutes; well-known examples are hayfever, asthma, and hives.
Immwtoglobulins	Antibodies
in vitro	Measured under artificial conditions in the laboratory--e.g., in a peal dish or test tube; as opposed to in vivo, in the body.
Leukocytes	White brook cells. See lymphocytes.
Lymphocytes	The majority of leukocytes are lymphocytes, white blood cells that are important for making antibody, as well as specifically taking part in surveying for and eliminating tumor cells, and cells carrying infectious agents.
Lymphokines	Cell products that serve as chemical mediators of lymphocyte functions.
Mitogens	Substances that induce lymphocyte proliferation.
Natural killer (NK) cells	Cells that are thought to provide an important defense against cancer and virus-infected cells.
Suppressor Tlymphocytes	Act to shut off helper Tlymphocytes when sufficient antibody has been produced.
Tlymphocytes	Thymus-derived lymphocytes, critical to the functioning of the cellular immune response.

example, a virus, a bacterium, portions or products of viruses or bacteria, and allergens can act as antigens.

The organs of the *immune system* include the thymus, bone marrow, lymph nodes, spleen, tonsils and adenoids, and Peyer's patches (the latter are located in the small intestine). Collectively, these "lymphoid" organs play roles in the growth, development, and deployment of lymphocytes (white blood cells).

The two major arms of the immune system are the *humoral* immune system and the *cellular* immune system. In the former, *B-lymphocytes* produce *antibodies* or *immunoglobulins-scrum* proteins that are induced by and react with antigens with exquisite specificity. The humoral immune response is important for defense against bacteria and viruses in body fluids.

The *cellular immune response,* the non-antibody-producing arm of the immune system, is important for defense against intracellular viruses, transplanted tissue, cancer cells, fungi, and protozoans; it has cells that move around the body that can kill such target cells. *T-lymphocytes* (thymus-derived lymphocytes), the lymphocytes crucial for cellular immune system function, have a number of subgroups. For example, *cytotoxic T-lymphocytes* migrate to the invasion site in the body, attach themselves to cells expressing foreign antigens, and produce cytotoxic factors that destroy the cells.

livo T -lymphocyte subpopulations have particular importance because of their regulatory effects on immunity. *Helper T-cells* stimulate B-lymphocytes to produce antibody, whereas suppressor *T-lymphocytes* act to shut off helper T-cells when sufficient antibody has been produced. Significant disturbances in the helper/ suppressor cell ratio can have important health consequences. Low ratios are found in patients with immunodeficiency disorders. In contrast, high helper/suppressor cell ratios are found in some naturally occurring autoinunune diseases-for example, systemic lupus erthyematosus, hemolytic anemia, severe atopic eczema, and inflammatory bowel disease; in these cases, the immune system appears unable to discriminate self from nonself, and attacks the body's own cells. The loss of suppressor cells may occur for a number of reasons, including their destruction by autoantibodies (antibodies made against self).

Lymphocytes and other kinds of blood cells can synthesize *lymphokines* (also called cytokines)-essential chemical mediators of various aspects of the immune response. The *interferons* (1FNs) and the *interleukins (ILs)* are two broad groups of cytokines.

Another component of the cellular immune response, *natural killer* (NK) cells, serve a vital immunological function: They defend against cancer and virus-infected cells. NK cells form an antitumor surveillance system, and appear to be critical in prevention of tumor growth and metastases as has been shown in animal models (Whiteside, Bryant, Day, & Herberman, 1990). They are labeled "natural" killers because they do not require prior exposure to a particular antigen and can therefore kill target cells in a nonspecific manner.

The central nervous system (CNS) and the immune system can communicate through multiple pathways. *Hormones* are very responsive to certain emotional states, and hormones can mediate immune function (e.g., see Baum & Grunberg, Chapter 8, this volume; Ader et al., 1991; Malarkey, Kiecolt-Glaser, Pearl, & Glaser, 1994). In addition, there may be direct connections as well; for example, nerve terminals have been found in the spleens of rats that are in physical contact with T lymphocytes (Ackerman, Bellinger, Felten, & Felten, 1991).

These CNS-immune system interactions can produce rapid changes, as shown in a recent series of studies of acute laboratory stressors (using human subjects) that generally last a half hour or less (reviewed in Kiecolt-Glaser et al., 1992). In contrast to the decrements in lymphocyte numbers reported in some studies of

longer-term naturalistic stressors (Herbert & Cohen; 1993b), acute laboratory stressors appear to increase cell numbers in some lymphocyte subpopulations. One possible mechanism may be the acute secretion of stress-responsive hormones, particularly *catecholamines,* which can alter a number of aspects of immune function (Rabin et al., 1989).

In fact, the immunological changes observed following short-term stressors are very similar to those that have been described following epinephrine injections (Crary et al., 1983x, 1983b). These epinephrine-induced changes are thought to reflect transient alterations in lymphocyte migration from lymphoid organs and peripheral blood mediated through receptors on lymphocytes or via the sympathetic nervous system innervation of lymphoid organs like the spleen (Ackerman et al., 1991; Crary et al., 1983x, 1983b; O(taway & Husband, 1992); patients whose spleens have been removed show much smaller changes in response to an epinephrine infusion than do normal subjects (Van Tits et al., 1990). These latter studies are critical to the interpretation of immunological data from acute laboratory stressors; that is, transient alterations in lymphocyte subpopulations are thought to reflect simple changes in the distribution of cells in circulation in peripheral blood (a process called "trafficking"), not a real change in cell numbers.

Immunological Assays: Basic Information

In order to measure different aspects of immune function, the numbers and/or functional abilities of subgroups of leukocytes (white blood cells) are assayed in blood samples. Immunological assays can be roughly divided into two categories. *Functional assays* reflect the "performance" or the functional efficacy of the cells. In contrast, *enumerative assays* provide information on percentages or numbers of cells. Cell numbers and cell function are not necessarily correlated (e.g., cells may not be differentiated or activated).

A number of leukocyte subpopulations perform specialized immunological functions, and no single immunological assay provides a global measure of immune system function; for this reason, PNI studies typically include a battery of assays. However, because of the interdependence of the various components of the immune system, adverse changes in one subpopulation of lymphocytes, for example, may produce multiple, cascading effects. There are numerous ways to measure various aspects of immune function, and the number of assays is steadily increasing as technology develops. In this section, we limit our discussion to some of the most common assays used in human PNI studies; the references listed in the introduction provide much more extensive information on the range of assays and their interpretation.

Enumerative Assays

Quantification of lymphocyte subpopulations most commonly involves the use of monoclonal antibodies that are directed at specific

counted by an instrument called a flow cytometer. For example, if the CD4 monoclonal antibody has been used, then the *percentage* of helper/inducer T-cells within a sample can be assessed. In addition, an absolute lymphocyte count, available as part of a complete blood count (CBC), is needed to convert the percentage of CD4 cells into the *absolute number* of CD4 cells that represent one prognosticator in human immunodeftciency virus (HIV) infection (see Lopez, Fleisher, & deShazo, 1992).

Collection of concurrent CBC data should be routine when lymphocyte percentages are being assessed; data from two meta-analyses suggest that cell numbers may be more strongly related to stressor appraisal or depression than are cell percentages (llerbert & Cohen, 1993x, 1993b). (It should be noted that CBC data generally do not, by themselves, provide useful data for PNI studies since simple lymphocyte counts are not particularly informative in "normal" populations.)

Functional Assays

In our experience, functional assays are more strongly and reliably related to psychological stressors than are enumerative assays (e.g., Kiecolt-Glaser & Glaser, 1991). In addition, functional assays are essential when one is studying older adult populations, as will be discussed shortly.

Blastogenesfs. Lymphocytes are normally found in a resting, nonreplicative state. In order to react to an infection and induce protection, cells need to be activated to replicate and to produce high levels of cytokines. When the immune system has identified and processed an antigen, both T and 13-lymphocytes are induced to proliferate and differentiate into functional subpopulations. A given antigen will stimulate this sequence for only the small subset of cells that have specific compatible receptors; all lymphocytes carry surface receptors that recognize one specific antigen, like a lock and key.

However, the in vitro use of mitogens (substances used in the laboratory that have the ability to stimulate lymphocyte proliferation or replication for large subsets of lymphocytes; analogous to a master key) can provide information on the immune system's ability to respond to certain foreign substances. The proliferative response of both T and B-lymphocytes to stimulation by *mitogens* (termed *blastogenesis)* such as phytohemagglutinin (PHA, which stimulates T-cell proliferation), pokeweed mitogen (PWM, which stimulates both T and B-cells) and concanavalin A (Con A, another Tcell mitogen) is thought to provide a model of the body's response to challenge by infectious agents such as bacteria or viruses (Reinherz & Schlossman, 1980).

Typically, blastogenesis involves incubation of lymphocytes with a mitogen in tissue culture media that includes a radioactive isotope. As lymphocytes replicate, they incorporate the isotope into cellular DNA. Proliferation (cell division) can be quantified by an instrument that measures the emission of radiation expressed in "counts per minute" (cpm), thus providing a measure of radioisotope uptake or utilization as a function of cell division-that is, the response to the mitogen/ antigen.

Blastogenesis is one of the few immunological assays that has been reliably

associated with relevant health parameters. Decreased lymphocyte proliferation reflects the down-regulation of normal immune responses in a variety of immunodeficiency conditions, including acquired immune deficiency syndrome (AIDS) (Fletcher, Baron, Asntan, Fischl, & Klimas, 1987), as well as less severe illnesses (e.g., see Cogen, Stevens, Cohen-Cole, Kirk, & Freeman, 1982; Lumino, Welin, Ifirvonen, & Weber, 1983), and even normal aging (Roberts-Thompson, Whittinghant, Youngcliaiyud, & MacKay, 1974).

Because two common mitogens, Con A and PHA, are inexpensive and easily obtained, they are used routinely to induce T-lymphocyte proliferation. They do this in a nonspecific way that does not involve the T-cell receptor. Mitogens are used to study T-cell responses. However, it is known that *antigens* induce T -cell proliferation by binding (specifically) to the Tcell receptor; when this happens, the T-cell is triggered to undergo cell division. A monoclonal antibody to the T3 receptor provides another way to measure T-cell proliferation. When this monoclonal antibody binds to the T-cell receptor, it, too, induces cell division in a manner similar to that induced by an antigen (e.g., Kiecolt-Glaser et al., 1993).

NK Cell Activity. A number of PNI studies have assessed the ability of NK cells to lyse or destroy "target" cells (usually cells from a tumor cell line), a process referred to as NK lysis (e.g., Kiecolt-Glaser, Garner, Speicher, Penn, & Glaser, 1984; Kiecolt-Glaser et al., 1985, 1993). To measure NK cell activity, target cells are grown in a media that is supplemented with a radioisotope; they incorporate the isotope into the cytoplasm of the cell. When NK cells are subsequently incubated with these specially prepared target cells, they lyse the target cells, releasing the isotope; the efficacy of lysis or killing is determined by measuring the amount of isotope released in the process. NK cell activity has a moderate to large effect size in various "stress" or depression studies (Herbert & Cohen, 1993a, 1993b).

Latent Herpesvirus Antibody Titers. The immune system "remembers" pathogens it has previously met, and immunological memory is an important principle that underlies the success of vaccination programs. For example, once a person has been exposed to an infectious agent (erg., poliovirus) the immune system mounts a defense and eradicates the invading agent; thereafter, that person is very unlikely to develop a clinical illness associated with that infectious agent again because the immune system can quickly destroy the agent should he or she be reexposed. I lowever, some viruses are capable of hiding in a latent state within specific host cells and thus can escape destruction by the immune system; HIV and the herpesviruses provide notable examples. Assays that reflect alterations in herpesvirus latency appear to be quite sensitive to a variety of stressors (Glaser & KiecoltGlaser, 1994a; Kasl et al., 1979; Kiecolt-Glaser et al., 1993; McKinnon, Weisse, Reynolds, Bowles, & Baum, 1989)

Individuals will remain latently infected for life after infection with a herpesvirus. The competence of the cellular immune system is thought to be a critical factor in controlling the primary herpesvirus infection, as well as subsequent control of virus latency (Glaser & Kiecolt-Glaser, 1994a). In

undergoing immunosuppressive therapies as in organ transplants, the immune SN'stern's control over latent herpesvirus replication is impaired. In these cases, reac

tivation of latent herpesviruses can occur and may result in disease. Furthermore. there are also characteristic elevations in herpesvirus antibody titers that can occur in the absence of any symptoms (Glaser & Kiecolt-Glaser, 1994a). These elevations in herpesvirus antibody titers are thought to reflect the memory antibody response to increased synthesis of the virus or virus proteins. When the cellular immune system becomes more competent (e.g., after cessation of immunosuppressive therapies). there are normally decrements in herpesvirus antibody titers. Thus, although the conclusion seems counterintuitive, *higher* antibody titers to latent herpesviruses suggest that the cellular immune system is *less competent* in controlling herpesvirus latency (Glaser & Kiecolt-Glaser, 1994a).

Antibody titers to latent herpesviruses show reliable changes in response to psychosocial stressors in asymptomatic individuals, particularly Epstein-Bare virus (EBV) and herpes simplex type t (HSV-I) (e.g., Glaser, Kiecolt-Glaser, Speicher. & Holliday, 1985; Glaser et al., 1987, 1991; Kiecolt-Glaser et al., 1985, 1991. 1993); in fact, herpesvirus antibody titers have shown the most consistent relationships to psychosocial variables of any of the diverse immunological assays we have used in our laboratory. In accordance with the suggestion that elevations in herpesvirus antibody titers reflect a broader down-regulation of cellular immune function, we have found also that specific T-cell killing of EBV-infected target cells decreased, synthesis of an induced lymphokine was altered (Glaser et al., 1987), and the proliferative response to several EBV polypeptides (viral proteins) also decreased in association with stress (Glaser et al., 1993).

Other Functional Assays. We have limited our descriptions to some of the most commonly used functional immune assays. Although an extended discussion of less routine assays is not possible in this chapter, investigators have also found stressrelated differences in such diverse aspects of immune function as antibody and virus specific T-cell responses to a viral vaccine (Glaser, Kiecolt-Glaser, Bonneau, Malarkey, & Hughes, 1992; Jabaaij et al., 1993); the production of two key lymphokines, gamma interferon and interleukin-2 (IL-2) (Glaser et al., 1987, 1990); alterations in the expression of a receptor for IL-2 on the surface of lymphocytes; and modulation of IL-2 receptor gene expression (Glaser et al., 1990)

Choosing Appropriate Immunological Assays

Investigators interested in the role that various stressors may play in the incidence, severity, or duration of infectious diseases, cancer, immunodeficiency diseases. or autoimmune diseases might be interested in certain aspects of immune function in their subjects. Alternatively, if a researcher simply wants some kind of "biological" marker to supplement

question is determined by the experimental questions of interest, the expertise and interests of one's collaborating immunologist, the populations studied, the amount of blood that can be drawn from each subject, available funds, and other logistical constraints.

Collaborating with an Immunologist

The development of a collaborative relationship with an immunologist is the most critical element for a behavioral scientist who wishes to begin PNI research. The immunologist will help design studies with an eye to the methodological and logistical constraints discussed in this chapter, choose assays that are appropriate to the study population that can be performed in his or her laboratory, review immunological data from the study to ensure both reliability and validity, and provide immunological expertise necessary for the interpretation of results. The behavioral scientist might also form a collaborative relationship with a physician whose clinical specialty involves immunologically mediated disorders (e.g., AIDS or asthma); such a person is likely to be knowledgeable about both clinical issues and associated immunological alterations.

Immunologists represent a diverse group of biological scientists who generally Nave a relatively narrow focus for their own work and interests. The skills, training, rnd resources of an immunologist strongly influence his/her choice of assays for Pint efforts with a behavioral scientist: for example, cytokine researchers are likely i) see cytokine assays as central to understanding alterations in immune function, whereas a herpes virologist/ immunologist might argue that assessing aspects of Yerpesvirus latency provides a window on the competency of the cellular immune ~rsponse (e.g., Glaser & Kiecolt-Glaser, in 1994a). Thus, the immunologist must ~e a key player in choosing the type of assay; by way of analogy, a behavioral +cientist should not expect a neuropsychologist to have any enthusiasm or expertise for a collaborative venture if the scientist insists on using the Rorschach as the project's primary measure of cognitive function.

Sometimes it is not possible to establish a collaborative relationship with an ntmunologist. Hospital or commercial laboratories will perform many immunological assays on a fee-for-service basis, albeit generally at much greater cost than with r collaborator. If a collaborative relationship is not possible, an immunological consultant who reviews raw data can provide helpful input on the multiple technical ?roblems that are not obvious to an untrained eye, as well as guidance in the nterpretation of results.

Matching Immunological Assays to Specific Research Questions

~s mentioned earlier, a wide range of immunological functions appear to be stressesponsive; given the limited knowledge about which immunological alterations n:ry be connected to actual health changes, there are no clear guidelines for assays 'tat may be "essential" beyond those that have clear relevance to the health status of

-11 the behavioral scientist's proposed population and research questions. For example, the number of CD4 (helper/ inducer) T-lymphocytes is one key marker for HIV progression (Lopez et al., 1992). The function and numbers of NK cells (particularly their function) appear relevant to a number of cancers, particularly the spread of metastatic cancer (Whiteside et al., 1990). The number of suppressor cells and the helper/suppressor ratio provide important information for certain autoimmune diseases (e.g., systemic lupus erthyetnatosus, hemolytic anemia, severe atopic eczema, and inflammatory bowel disease); in these diseases, the loss of suppressor cells may correlate with clinical severity (Reinherz & Schlossman, 1980). Vaccine studies normally involve the assessment of antibody production and a T-cell and/or cytokine response to vaccine antigens (Glaser et at., 1992; Jabaaij et al., 1993). Researchers who are infecting subjects with a specific virus (e.g., a cold virus) need to measure antibody to specific viral antigens both before and after infection, as well as aspects of the cellular immune response such as the virus-specific T-lymphocyte response.

Age-related immunological decrements are thought to be associated with the greatly increased morbidity and mortality from infectious illness observed in the elderly; for example, mortality from influenza infection is four times greater among people who are over 60, compared to those younger than 40 (Bums. Lum. Seigneuret, Giddings, & Goodwin, 1990). These age-related immunological changes are demonstrated in the functional aspects of the cellular immune response. but only minimally or not at all with respect to cell numbers (Murasko. Weiner. & Kaye, 1988; Wayne, Rhyne, Garry, & Goodwin, 1990). Thus, functional assays are the highest priority when the health of the elderly is of interest.

As mentioned earlier, no single immunological assay provides a global measure of immune system function; thus, researchers should utilize several different assays to assess different aspects of immune function. In addition to choosing assays based on population-specific questions, researchers may also wish to consult two metaanalyses that review the relationships among stress depression. and immune function. Herbert and Cohen (1993a, 1993b) describe the effect sizes for a number of common immunological assays; these articles provide helpful information for choosing more psychosocially "sensitive" assays, as well as data on which to base the number of subjects needed to detect certain effects.

Immunological Data and Health Status: Infectious Illness

It is sometimes erroneously assumed that stress-related alterations in immune function translate directly into changes in health, and that immunological data can serve as a surrogate measure of health status. In fact, the extent to which relatively small immunological changes affect the incidence, severity, or duration of immunologically relevant diseases is unknown. The type, intensity, and chronicity of a stressor; the degree and pervasiveness of immune modulation; and an individual's prior

ships between poorer immune function and illness, particularly infectious illnesses. Infectious illnesses occur relatively infrequently in the general population, with most adults reporting only a few illness episodes a year. As a consequence, alterations in low base rates are difficult to detect, particularly with the relatively small sample sizes necessitated by the time and expense inherent in PNI research. Moreover. exposure to pathogens is essential for development of infection, but exposure is not simply a random event; for example, families with small children are likely to have a higher incidence of illness, whereas socially isolated individuals are less likely to be exposed to pathogens.

Alternatively, in order to demonstrate causal relationships between psychooocial stressors and the development of infectious illness, investigators have inoculated subjects with a pathogen, a vaccine, or a harmless antigen. By evaluating the timing and strength of antibody and T-cell or cytokine response following inoculation, a researcher may model the body's response to infection. For example, we gave each of a series of three hepatitis B vaccine inoculations to 48 medical students on the last day of three 3-day examination series to study the effect of an academic stressor on the students' ability to generate an immune response to a *primary* antigen-that is, an antigen to which they had no previous exposure (Glaser et al ., 1992). A quarter of the students *seroconverted* (produced an antibody response to the vaccine) after the first injection, and they reported feeling less stressed and less anxious than those students who did not seroconvert until after the second injection. In addition, students who reported greater social support demonstrated a stronger immune response to the vaccine at the time of the third inoculation, as measured by antibody titers to one hepatitis B antigen, and the tilastogenic response to one of the hepatitis B viral peptides (proteins). These stressrelated alterations in hepatitis B vaccine response have subsequently been replicated by another laboratory (labaaij et al., 1993). These data suggest that the immunological response to a vaccine can be modulated by a relatively mild stressful event in young, healthy adults.

Vaccine response data such as these can provide a window on the body's response to other pathogens, such as viruses or bacteria; individuals who show a delayed or blunted vaccine response could be at greater risk for more severe illness.

Research with older adults provides further support for these assumptions. Many older adults do not respond to vaccines (or other "new" antigens) as efficiently as younger adults (Phair, Kauffman, Bjornson, Adams, & Linnemann, 1'178). Older adults attain lower peak antibody levels after vaccination, and show more rapid or steeper rates of decline than do younger adults in their immune response to influenza and other antigens (Burns et al., 1990). These age-related immunological decrements are thought to be associated with the greatly increased morbidity and mortality from infectious illness in the elderly; for example, among adults over 75 years of age, pneumonia and influenza together are the fourth leading cause of death (Yoshikawa, 1983). Thus, this same age group shows poorer vaccine responses and greater morbidity and mortality from infectious illnesses.

Researchers have also inoculated subjects with a live virus. Cohen, Tyrrell, and Smith (1991) prospectively studied the relationship between stress appraisals and susceptibility to colds by inoculating volunteers with five

in which subjects are inoculated with pathogens or vaccines provide researchers with a means of controlling exposure and concentration of a pathogen: moreover, because immune function may be assessed prior to the infectious challenge, these studies provide better data on causality than is possible with naturally occurring infections.

Intervention Studies

Researchers who wish to try to enhance immune function via an intervention need to consider the initial immunological status of their potential subjects. If an individual's immune system is functioning satisfactorily, it may not be possible to "enhance" immune function above normal levels; in fact, it is possible that it would be undesirable to do so. More is not necessarily better; for example, an overactive immune system may lead to autoimmune disease. In the absence of any age-, disease- or stress-related downward alterations in a study population's immune function, any intervention designed to enhance immune function could fail to alter immune function because of homeostatic regulation; if effective in enhancing immune function, it could be maladaptive.

Changes Following "Laboratory" or Other Brief Stressors

Responses to acute laboratory stressors show considerable variability among individuals and across situations. Individual differences in cardiovascular reactivity have been studied extensively (see Chapter 9 for details; also see Manuck, Kasprowicz, Monroe, Larkin, & Kaplan, 1989); since cardiovascular and catecholaminergic reactivity tend to co-vary when assessed under the same conditions, researchers have analyzed immunological changes in relationship to cardiovascular reactivity (Bachen et al., 1992; Manuck, Cohen, Rabin, Muldoon, & Bachen, 1991; Sgoutas-Emch et al., 1994). High-reactivity subjects demonstrate greater immunological change than low-reactivity subjects, with the latter showing little or no change (reviewed in Kiecolt-Glaser et al., 1992). Both the duration and intensity of psychological stressors (as indexed by cardiovascular changes) are related to the breadth and magnitude of immune changes in laboratory studies. Obviously, at a minimum, heart rate and blood pressure measurements (and, ideally, plasma catecholamines) are needed to aid comparisons across studies with various acute laboratory stressors.

Logistic Issues In the ideal study, blood samples would be collected from all experimental and control subjects at precisely the same time. The realities of research normally make such a plan impossible. When samples are collected on multiple days from groups of subjects who are hypothesized to differ on some characteristic, blood samples

from ,the different subject cohorts need to be assayed simultaneously (e.g., rather than samples being assayed from depressed subjects on one day and nondepressed control subjects on the next). The day on which samples are gathered and assays performed (i.e., the "measurement occasion") can account for as much as 97 percent of between-group differences, depending on the assay and the laboratory (Schleifer, Keller, Bond, Cohen, & Stein, 1989). In order to avoid systematic bias, it is important to intermingle subjects from various groups when samples are collected. In addition, the immunological data obtained from "control" subjects run on the same day as the targeted population can be used statistically to control for daily variation using analysis of partial variance (Schleifer, Haftan, Cohen, & Keller, 1993).

A related issue is the length of time during which blood samples sit before assays are begun in the laboratory. Sample storage time can have significant effects on some immunological parameters (Fletcher et al., 1987). This becomes a particularly important issue when samples are sent to a commercial laboratory, since laboratories may assay samples immediately on some days, but wait up to 24 hours (or more) on other days, since they try to cluster assays to conserve technician time. The error variance related to differences in storage time is likely to be much greater in commercial laboratories. Unless samples can be frozen (and preparation is required before freezing), shipping samples to a distant laboratory will also introduce these same difficulties with variability in time.

All subjects need to be studied at the same time of day (i.e., within the same 1to 3-hour window, depending on the parameters of interest) to minimize error variance associated with diurnal variation. A few assays, which have longer halflives, are quite stable so that diurnal variation is not problematic: In particular, the half-life for immunoglobulins (antibodies) is 6 to 8 days for IgA, 9 to 11 days for IgM. and 21 days for IgG.

Immunological assays require considerable time and technical skill. For example, setting up routine assays using blood samples from several individuals generally takes an experienced technician the better part of a day; most of the common assays described in this chapter require fresh cells, which means that blood samples cannot be drawn and then frozen for later processing. Thus, if blood samples arrive at an immunology laboratory in the afternoon, the technician will then need to work through the evening or later. Within limits, however, the time per subject is not additive, and it may not require substantially more time to prepare several samples for the same assay than a single sample. In addition to the savings in technical time, the grouping or clustering of samples is highly desirable because of the "measurement occasion" issues described above.

In order to obtain a sufficient quantity of blood, samples are drawn from the *arm;* depending on the battery of assays in a particular study, we generally draw 30 to 60 cc (1 to 2 ounces). Although 30 to 60 cc is a small fraction of an adult's total blood volume, it means that a phlebotomist or nurse will need to fill three to six 10cc tubes, a process that disturbs some subjects much more than others. We always mention that blood draws are a key part of the study when recruiting subjects, and we ask potential subjects if they have any needle phobias; attempting to draw blood from a needle phobic presents a number of obvious problems, particularly fainting.

5 When repeated blood samples are collected over a period of several hours, use of an indwelling catheter avoids the additional distress and pain produced by repeated venipuncture. Adaptation periods of 30 minutes or more are advisable following catheter insertion (Manuck et al., 1989). The amount of blood that can be obtained from subjects sets limits on the kinds and numbers of immunological assays that can be performed. Very few PNI researchers have attempted to study children or adolescents, in part because of the difficulties involved in obtaining blood samples; in addition, they are likely to have a higher incidence of needle phobias.

One assay, secretory IgA (s-IgA), uses saliva, rather than blood. However, salivary flow changes in response to stressors, providing an additional methodological problem. Studies that use s-IgA need to control adequately for flow rate because of the associated methodological problems that may otherwise make interpretation of such data questionable (Herbert & Cohen, 1993b; Stone, Cox, Valdimarsdottir. & Neale, 1987).

PNI research is expensive, and supplies for a small pilot study can easily cost several thousand dollars, aside from the labor needed for the assays. In addition to the materials themselves, there may be other, less obvious costs; for example, radioisotopes, used in a number of assays including blastogenesis and \K cell lysis, incur expensive charges for disposal of isotope waste. Hospital laboratory charges, although including both supplies and labor, may be prohibitive; for example, analysis of a single blood sample for one assay, NK cell lysis, may cost from $200 to $400.

In the beginning of a particular study, one should buy sufficient quantities of laboratory supplies for the entire study if at all possible (e.g., mitogens, fetal bovine serum, media, plasticware, etc). Although this suggestion may seem trivial or commonsensical, it can have enormous consequences for laboratory data in a given study. For example, we found a 10-fold difference in the relative values obtained for gamma interferon using different lots of Con A in two studies in which lymphocytes were stimulated with Con A to produce gamma interferon (Glaser, Rice, Speicher, Stout, & Kiecolt-Glaser, 1986; Glaser et al., 1987). Thus, if an investigator were to buy different batches of mitogen without attempting to examine their relative potencies, one could easily show remarkable artifactual changes over time. For these same reasons, assays need to be conducted within the same laboratory across a study.

A number of immunological assays do not have "nonnal" values or ranges for comparison purposes, particularly the functional assays. Moreover, functional assays appear to show greater day-to-day variation than enumerative assays (Schleifer et al., 1993). In addition, protocols may vary from one laboratory to another, and differences in the methods used for immunological assays can produce dissimilar data. For example, the length of time that lymphocytes are routinely incubated with a mitogen may be 48 hours in one laboratory, 72 hours in another; longer incubation times are likely to result in higher "counts per minute"-that is, greater uptake of the radioisotope-while not reflecting real differences in the proliferafve response. Some functional assays are typically run in triplicate, and the triplicates are then averaged (e.g., blastogenesis, NK cell lysis)-providing one indication of .he inherent variability of the assay.

As noted earlier, distressed individuals are more likely to Gave life-styles that put them at greater risk, including poorer health habits such as a greater propensity for alcohol and drug abuse, poorer sleep, poorer nutrition, less exercise, etc.; and these health behaviors may make immunological data much more variable (see review in Kiecolt-Glaser & Glaser, 1988). For this reason, careful assessment of health-related behavior is essential. By making such assessments a routine part of any protocol, it may be possible statistically to control some of the error variance related to these factors, thus providing a clearer understanding of psychosocial influences on immune function.

Future Directions

Convergent evidence from several laboratories suggests that chronic stressors may enhance differences in sympathetic nervous system (SNS) reactivity, neuropeptide release, and immune function (Fleming, Baum, Davidson, Rectanus, & McArdle, 1987; Irwin et al., 1992; Kiecolt-Glaser et al., 1992; McKinnon et al., 1989). If sympathetic activation is a marker or determinant of immune function, then longitudinal studies that evaluate the relationships among psychosocial stressors, SNS activity and reactivity, stress-related immune and endocrine changes, and longerterm changes in health are needed to determine whether extrapolations from crosssectional data on acute events to chronic and longitudinal effects are warranted. Through interdisciplinary collaborations, the measurement of multiple biological stress responses should help investigators understand how psychosocial stressors get translated into adverse health changes.

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