Neural and Pavlovian Influences on Immunity

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Abstract—The traditional view that the nervous and immune systems are functionally independent (aside from general stress effects and autoimmune disorders of the nervous system) is being challenged by a new view that the nervous system regulates the activity of the immune system. If this is true, it should be possible to change the activity of the immune system by means of Pavlovian conditioning, just as it is possible to condition other physiological events influenced by the autonomic nervous system or neuroendocrine substances. Evidence for autonomic and neuroendocrine modulation of immune activity is briefly reviewed; and, the various studies reporting conditioned immune effects, the physiological mechanisms most likely involved, and their possible significance are discussed.

FOR THE BENEFIT of readers unfamiliar with immunology, a very brief introduction to some concepts and terminology must first be given. The immune system comprises a diverse set of blood cells and complex molecules with various activities. Its function is to recognize "self" from "nonself," and to attack and inactivate whatever is perceived to be "nonself." Vertebrate immune systems are remarkable for their specificity, their extensive repertoire of distinct responses, and their capacity for memory. "Memory" is a consequence of the proliferation and retention of cells with the desired specificity of reaction.

In the discussion to follow, three forms of immune responses are predominant. The first is antibody production. Immunology is founded on the circular definition that antibodies are immunoglobulin molecules made in response to, and that specifically react against, antigens. This response is commonly measured either by observing the highest dilution at which sera will agglutinate a fixed amount of antigen (the "titer"), or by counting the number of splenic cells producing antibodies in a "plaque assay," in which the splenic cells are immobilized in a gel with target cells that lyse when the antibodies attach to them. Thus, each cell producing antibodies of the correct specificity is eventually surrounded by a plaque of lysed target cells. This latter technique distinquishes between antibodies of two classes, IgG (class G immunoglobulin) and IgM (class M immunoglobulin), which differ in size, but not necessarily in their specificity of attachment to the antigen.

The cells that make antibodies come from a subset of lymphocytes called B cells. Like other subsets of lymphocytes, they share some surface markers, but differ in the specificity of their receptors for antigens. When a new antigen is encountered, only those B cells with a receptor which recognizes the antigen will proliferate, and then produce antibodies. The antibodies produced share the same antigenic specificity as the receptors on the surface of the B cells. Antibody production for some antigens requires the assistance of other lymphocytes, T cells ('T' for thymus derived, the organ needed for their maturation); such antigens are referred to as "T cell dependent," to distinguish them from those that are "T cell independent"—Sheep red blood cells are a commonly used T cell dependent antigen. *In vitro*, human T cells have the curious property of nonspecifically forming rosettes with sheep red blood cells.

Another immunologic response to be encountered in this review will be the lysis of cancer cells by "natural killer cells." These cells may play an active role in immune surveillance against tumor cells. Their activity can be measured *in vitro* by recording the release of radioactive label from lysed target cells, or in *in vivo* by observing the survival time and percentage in animals inoculated with tumor cells.

Similarly, allogenic cells (cells from genetically different members of the same species) are lysed directly by cytotoxic T lymphocytes. As in antibody production, this occurs with great specificity. This response is measured also by the release

of radioactive label from target cells (in mixed lymphocyte cultures).

Three other responses also will be mentioned: "antibody-dependent cell-mediated cytolysis," in which antibodies provide for the specificity of response; delayed hypersensitivity, a cell-mediated reaction seen in positive tuberculin skin tests; and, anaphylactic shock, which involves the release of histamine and other physiologically active substances from cells bearing IgE antibodies.

Some terms will be used frequently: lymphokines, which are substances made by lymphocytes that affect the activity of other lymphocytes rather than attacking the antigen; mitogens, which induce the proliferation of subpopulations of lymphocytes irrespective of the specificity of their antigen receptors; and adjuvants, which nonspecifically enhance antibody production when administered with antigens.

Finally, the immunosuppressive effects of stress traditionally are thought to be mediated by serum corticosteroids from the adrenal gland; this occurs in response to release of adrenocorticotropin (ACTH) by the pituitary (Keller, Weiss, Schleifer, Miller, and Stein 1983 Riley 1981).

This is, of course, a very brief and simplified introduction to immunology. However, the points above are sufficient to allow the reader to appreciate the complexity of autonomic, neuroendocrine, and conditional modification of immune responses.

Autonomic and Neuroendocrine Immunomodulation

In addition to nonspecific hormonal (Ahlquist 1976) and stress effects (*e.g.*, Solomon, Amkraut, and Kasper 1974, Riley 1981), there appears to be neural activity which is specific for the modulation of immunity (Amkraut and Solomon 1975, Besedovsky, del Rey, and Sorkin 1983 a and b; Besedovsky and Sorkin 1977, 1981, Fauman 1982, Korneva, Klimenko, and Shkhinek 1978, MacLean and Reichlin 1981, Maestroni and Pierpaoli 1981, Pierpaoli 1981, Rogers, Dubey, and Reich 1979, Solomon and Amkraut 1981, Spector and Korneva 1981). Support for autonomic and neuroendocrine control of immunity has been obtained through the traditional methods of neuroscience.

1) Brain lesions, particularly in the anterior hypothalamus, produce impaired immune responses in experimental animals (Stein, Schiavi and Camerino 1976, Stein, Schleifer, and Keller 1981) and this has been observed also in human patients with brain tumors (Brooks, Netsky, Normansell, and Horwitz 1972, Brooks, Roszman, and Rogers 1976, Young, Sakalas, and Kaplan 1976).

2) Antigenic stimulation of the immune system affects neural activity in the hypothalamus (Besedovsky, del Rey, Sorkin, Da Prada, Burri, and Honegger 1983, Besedovsky, Sorkin, Felix, and Haas 1977, Broun, Mogutov, and Kan 1970, Korneva, Klimenko and Shkhinek 1974).

3) Electrical stimulation of the brain can increase immune activity (*e.g.*, Fessel and Forsyth 1963, Jankovic, Jovanova, and Markovic 1979, Korneva and Khai 1967).

4) Organs with important roles in immunity, such as the bone marrow, spleen, thymus, and lymph glands have innervation that is not associated with any muscle function, and sometimes is in close proximity to white blood cells (*e.g.*, Bulloch and Moore 1981, Calvo 1968, Fillenz 1970, Giron, Crutcher, and Davis 1980, Kuntz and Richins 1945, Reilly, McCuskey, Miller, McCuskey, and Meinke 1979, Sergeeva 1974, Williams and Felton 1981, Zetterstrom, Hokfelt, Norberg, and Olsson 1973). Local denervation of the spleen or neonatal sympathectomy with 6-hydroxydopamine results in elevated antibody responses (Besedovsky, del Rey, Da Prada, and Keller 1979, Miles, Quintans, Chelmicka–Schorr, and Arnason 1981, Williams, Peterson, Shea, Schmedtje, Bauer, and Felton 1981).

5) Neurotransmitter agonists and antagonists affect immunologic functions *in vivo* (Hall and Goldstein 1981), and receptors for neurotransmitters and putative neurotransmitters/neuromodulatory substances have been found on lymphocytes (*e.g.*, Eskra, Stevens, and Carty 1978, Gordon, Cohen, and Wilson 1978, Lappin and Whaley 1982, Richman and Wilson 1979, Roszkowski, Plaut, and Lichenstein 1977, Strom, Sytkowski, Carpenter, and Merrill 1974).

Each of the above methods merits some comment. There are conflicting reports on the effects of hypothalamic lesions on antibody production when an antigen such as egg albumin is injected (usually together with an adjuvant) and induces allergic sensitization. Ado and Goldstein (1973), Schiavi, Macris, Camerino, and Stein (1975), and Thrasher, Bernardis, and Cohen (1971) found no significant effect, although Filipp and Szentivanyi (1958), Macris, Schiavi, Camerino, and Stein (1970), and Tyrey and Nalbanov (1972) found decreased levels of antibodies. However, there is general agreement that hypothalamic lesions do protect the animals against lethal anaphylactic shock (Filipp 1973; Filipp and Szentivanyi 1958, Luparello, Stein, and Park 1964, Macris *et al.* 1970, 1972, Schiavi *et al.* 1975. The other papers only reported antibody levels).

Physiologic mechanisms, in addition to antibody production, are involved in anaphylactic shock (Amir 1984, Roy nd Karim 1983). Schiavi *et al.* (1975) suggested that the discrepancies in findings related to the level of antibody production may be related to the strength of the response; very high production might obscure the effect.

In addition to the above mentioned papers, there are reports of hypothalamic lesions suppressing antibody production (*e.g.*, Broun *et al.* 1970, Jankovic and Isakovic 1973, Korneva and Khai 1964). Jankovic and Isakovic (1973) also found suppression of delayed hypersensitivity. Recently, two groups demonstrated decreased natural killer cell activity after hypothalamic lesions (Cross, Markesbery, Brooks, and Roszman 1984, Forni, Bindoni, Santoni, Belluardo, Marchese, and Giovarelli 1983). Brooks, Cross, Roszman, and Markesbery (1982) found that rat spleen cell responsiveness to the mitogen concanavalin A decreased after lesions of the anterior hypothalamus developed, and increased after lesions in the mammillary bodies, hippocampus, and amygdala developed.

One problem in the interpretation of lesion studies is that the nervous system and cells of the immune system share antigenic cross-reactivity (Golub 1972, Jankovic, Horvat, Mitrovic, and Mostaric 1977, Oger, Szuchet, and Arnason, 1982, Reiff and Allen 1964, Schuller–Petrovic, Gerhart, Lassman, Rumpold, and Kraft 1983). Thus, surgery may, by disrupting the blood–brain barrier, expose antigens provoking an immune response that is directly and nonspecifically immunosuppressive, because antibodies attach to and interfere with the activity of lymphocytes. Brooks *et al.* (1972) found an immunosuppressive serum factor with the physical properties of antibodies in the blood of patients with intracranial tumors and depressed cell mediated immunity. Such a check for immunosuppressive antibodies should be incorporated into brain lesion studies.

An alternate interpretation of the brain lesion studies and of the studies on sympathectomy and neurotransmitter agonists/antagonists is that the animals are simply being stressed. Stress is not easy to control. It would be helpful to inquire whether the physiologic consequences of the lesions are similar to those seen in stress (*e.g.*, increased ACTH production resulting in immunosuppressive levels of corticosteroids), but this might lead one to dismiss something as a stress effect, although a nonstress-related regulatory role is also present. Stress may be a confounding influence in stimulation studies as well—analgesia or reinforcement may be reducing stress. These alternatives can be tested, but the problem of dismissing positive results still remains.

Electrical recording from neurons after immunization should be free of rival stress interpretations, but as a technique it is limited in its use because of the length of time in which immunologic reactions occur. In studying vision, one can look at the response of a single cell when light is flashed on and off in its receptive field. In the context of immunology, one is forced to compare the activity of neurons of immunized animals with the activity of neurons in nonimmunized animals. Therefore, one cannot readily determine the response characteristics of single neurons.

The major challenge for the concept of autonomic and neuroendocrine regulation of immunity is to prove that more is involved than simply very elaborate stress responses. Is this "regulation" really regulation in that immune responses are modulated other than as a consequence of psychological stress? One approach to this question is to study the physiology of communication between the immune and nervous systems; another is by means of Pavlovian conditioning. Work has been done in both areas.

Chemical Communication Between the Immune and Nervous Systems

As mentioned above, neurotransmitter agonists and antagonists affect immunologic responses. Cholinergic and alpha-adrenergic stimulation can increase B and T cell responses; beta-adrenergic stimulation can decrease these responses. Elevated dopamine may increase T cell responses but decrease B cell responses. Few neurotransmitters or distinctive responses (such as the few that were introduced at the very beginning) have been studied to know the full scope of possible control mechanisms (Hall and Goldstein 1981).

There are clinical observations that also implicate neural activity in the modulation of immunity. Schizophrenics who are not in remission and who are not receiving drug treatments have impaired immune responses, and psychiatric patients with depression almost always have allergies (Hoffer 1980, Solomon 1981). These may be examples of diseases of the central nervous system (CNS) producing further morbidity through abnormal autonomic or neuroendocrine control of immunity.

Besedovsky and his colleagues have published some interesting physiological studies of neuroendocrine modulation of immunity (see Besedovsky, del Rey, and Sorkin 1983 a and b for reviews). Blood corticosteroids rise to im-

munosuppressive levels during immune responses to antigens such as sheep red blood cells. Administration of lymphokine containing supernatants from immune cells stimulated *in vitro* by the mitogen concanavalin A to rats not previously given any immune stimulation results in an increased serum level of adrenocorticotropin (ACTH). Hypophysectomy (surgical removal of the pituitary) blocks this increase in ACTH. Besedovsky, del Rey, and Sorkin (1979) have suggested that such a neuroendocrine circuit may be responsible for antigenic competition between noncross-reacting antigens (*i.e.*, impaired immune function when several antigens are simultaneously presented). They have also found tht noradrenalin levels decrease in lymphoid organs upon antigenic challenge (Besedovsky, del Rey, Sorkin, Da Prada, and Keller 1979, del Rey, Besedovsky, Sorkin, Da Prada, and Arrenbrecht 1981), and that noradrenalin synthesis in the hypothalamus decreases during an immune response (Besedovsky, del Rey, Sorkin, Da Prada, Burri, and Honegger 1983). Blalock and Stanton (1985) found that lymphocytes secrete an ACTH-like substance; this might directly stimulate corticosteroid release and contribute to negative feedback in an immune-neuroendocrine regulatory circuit.

Another possible link between the central nervous system (CNS) and the immune system involves thymosins (hormones produced by the thymus gland), which have been detected in the CNS (Oates and Goldstein 1984). Thymosin alpha-1 stimulates a rise in serum corticosterone when injected into the cerebroventricular system of chronically cannulated mice (Oates and Goldstein 1984). Partially purified thymosin fraction 5, purified thymosin alpha-1, and lymphokine containing supernatants from concanavalin A stimulated spleen cells do not induce steroid output by rat adrenal fasciculate cells *in vitro*, but they do produce a transient increase in steroid hormones when given *in vivo* (Vahouny, Nyeyune-Nyombi, McGillis, Tare, Huang, Tombes, Goldstein, and Hall 1983); the CNS may be the mediator of this response.

Newborn and adult athymic (nude) mice have reduced levels of prolactin in the blood and high levels of luteotropic hormone; implantation of the thymus returns blood levels of these hormones to normal (Pierpaoli, Kopp, and Bianchi 1976). Blockade of adenohypophysis in athymic mice prevents the normal restoration of transplantation immunity when the animals receive thymus grafts (Pierpaoli *et al.* 1976). Therefore the thymus may be able to stimulate increased production of endocrine substances such as prolactin and gonadal steroids, which, in turn, may affect immunity (Grossman 1985). The role of the CNS needs further investigation.

Endorphins have potent but mixed effects on the immune system. In a recent review, Chang (1984) suggested that the diversity of endorphins and enkephalins, their receptors and subpopulations of immunoactive cells may account for the somewhat paradoxical findings that have been obtained. Lymphocytes apparently have two distinct receptors for beta-endorphin, one for the amino end of the molecule (this can be blocked by opiate antagonists such as naloxone and naltrexone) and the other for the carboxyl end (this is not affected by opiate antagonists) (Hazum, Chang, and Cuatrecasas 1979). Gilman, Schwartz, Miller, Bloom, and Feldman (1982) found that beta-endorphin enhances the proliferative response of rat lymphocytes to mitogens, while McCain, Lamster, Bozzone, and Grbic (1982) reported that beta-endorphin suppresses the mitogenic activity of human lymphocytes. Alpha-endorphin represents the first 16 amino acid residues of beta-endorphin and shares the amino end, but not the carboxyl end, of beta-endorphin. Alpha-endorphin does not affect the mitogenic activity of lymphocytes, but it does inhibit antibody production by means of an opiate-like receptor, which beta-endorphin does not (Johnson, Smith, Torres, and Blalock 1982). Wybran, Applebloom, Famaey, and Govaerts (1979) found that morphine stimulates, but methionine-enkephalin inhibits the rosette formation of T lymphocytes with sheep red blood cells.

Naltrexone at a high dose shortens the survival time of mice inoculated with lethal doses of neuroblastoma cells (Zagon and McLaughlin 1983), and both methionine–enkephalin and leucine–enkephalin administered to mice injected with murine leukemia cells increased the number of survivors (Plotnikoff and Miller 1983). Fatih, Liang, Murgo, and Plotnikoff (1984) also obtained increases in the activity of human natural killer cells incubated *in vitro* with either methionine–enkephalin or leucine–enkephalin. However, at very low doses, naltrexone increases the survival time of mice inoculated with neuroblastoma cells (Zagon and McLaughlin 1983).

Shavit, Lewis, Terman, Gale, and Liebeskind (1984) subjected rats to one of two inescapable footshock procedures, both of which induce analgesia, but only one by opioid mechanisms. They observed that survival time and percent survival after inoculation with tumor cells were lessened only by the opoid response, and that naltrexone blocked this suppression. It is important to consider stress and analgesia in such *in*

vivo studies because opiate antagonists may be acting directly on the surface of the lymphocytes, or indirectly by modulating the activity of the nervous system by increasing sensitivity to environmental stressors.

Human leukocyte interferon shares structural (antigenic) similarity with ACTH and endorphins (Blalock and Smith 1980). This suggests that endorphins may be able directly to affect immunity because of their similarity to interferon, and that interferon and perhaps some lymphokines may be able to stimulate the nervous system because of their similarity to endogenous opiates. Interferon has been found to enhance the excitability of cultured neurons (Calvet and Gresser 1979), and, when administered to morphine dependent rats, interferon treatment one hour prior to naloxone injection eliminates most of the behaviors associated with morphine withdrawal (Dafny 1983). Blalock and Stanton (1980) reported that noradrenalin induced an interferon-like antiviral state in mouse myocardial cells, but not in human amnion cells that were cultured separately *in vitro*. The addition of noradrenalin to cocultures of mouse myocardial cells and human amnion cells resulted in the development of antiviral activity in the human amnion cells, suggesting an interferon-induced transfer of viral resistance. Further evidence of common pathways of interferon and neuroendocrine activity comes from the finding that ACTH may suppress interferon production (Johnson, Torres, Smith, Dion, and Blalock 1984).

Blalock (1984) has obtained preliminary evidence suggesting that human lymphocytes produce substances that share structural similarities with hormones (based on immunofluorescence essays), and that the pattern of immunoreactive hormone production depends upon the antigenic stimulus used. This probably is not a result of different antigenic determinants per se, but rather reflects the involvement of different subsets of lymphocytes (*e.g.*, helpers, suppressors, cytotoxic T cells) in the natural response to particular antigens. The chemical communication between different cells within the immune system and between the nervous system and the immune system appears to be complicated, and biochemical analysis difficult because of the above-noted structural similarities.

Hemispheric Lateralization of Function and Immunity

Geschwind and Behan (1982) reported that persons who are lefthanded are more likely to have autoimmune disorders than those who are righthanded. This was attributed to the effects of testosterone on the development of the immune and nervous systems. Although this is very controversial (see Geschwind and Behan 1984, and Wofsy 1984 a and b, for further comments), it may be true for at least some autoimmune diseases (autoimmune disorders are a very heterogenous group) and, possibly, allergies.

Renoux, Biziere, Renoux, Guillamin, and Degenne (1983) found that after large ablations of the left neocortex in mice, splenic T cell numbers decreased in comparison to sham-operated and nonoperated controls, and that the cells themselves were less responsive in comparison to an equal number of control cells (number of IgG plaque forming cells and *in vitro* proliferation in response to the mitogen phytohemagglutinin). Lesions of the right neocortex did not alter significantly the number of splenic T cells, but their responsiveness (as measured above plus cytotoxic activity in mixed lymphocyte cultures) increased. Because they gave an eight-week recovery period between surgery and testing, the cortex may not be the main brain structure responsible for this balanced asymmetry.

Renoux *et al.* have extended their investigation of the immunologic effects of asymmetric cortical lesions, and incorporated the use of sodium diethyldithiocarbamate (imuthiol). This induces T cell maturation (expression of the Thy-1 surface marker) and function in athymic mice. Imuthiol has no significant influence on T cells *in vitro*, and has a serum half-life of approximately 20 minutes in mice. Heat stable fractions from imuthiol-conditioned liver cell cultures (but not from normal spleen, lymph node, or kidney cultures) were able to induce T cell maturation *in vitro* (Renoux, Renoux, Biziere, Guillaumin, Bardos, and Degenne 1984).

In one experiment, Renoux *et al.* (1984) administered either saline or imuthiol to mice previously given partial ablations of the left neocortex, partial ablations of the right neocortex, or sham lesions. The mice were killed four days after saline or imuthiol treatment, and the *in vitro* proliferative response of their T cells to alloantigens was measured in mixed lymphocyte assays. Imuthiol stimulated this response (relative to the saline-treated controls) in the mice with right neocortical lesions or sham lesions, but not in the mice with left neocortical lesions.

Similar experiments were performed on other immune responses: IgG and IgM plaque-forming splenic cells after injection of sheep red blood cells; lymphocyte proliferation in response to the mitogens concanavalin A and phytohemagglutinin; natural killer cell activity; and antibody-dependent cell-mediated cytotoxicity (Renoux *et al.* 1984). Only with the last response

was there no significant effect due to asymmetric lesions. Lesions of the left neocortex lowered natural killer cell activity and lesions of the right neocortex increased it. Imuthiol treatment did not significantly affect either natural killer cell activity or antibody-dependent cell-mediated cytotoxicity. Lymphocyte proliferation in response to mitogens was lower in the saline-treated mice with left neocortical lesions than in saline-treated mice with right neocortical lesions; imuthiol treatment eliminated this difference and produced a moderate response midway between the levels of the saline-treated asymmetrically lesioned animals. Similarly, the numbers of antibody-producing cells were lower in the saline-treated animals with left neocortical lesions than in their counterparts with right neocortical lesions. In this case, imuthiol increased the number of IgM plaque-forming cells in all groups and left no significant difference due to the lesions. The number of IgG plaque-forming cells increased after imuthiol treatment in the left-lesioned animals, but it remained well below the level of the saline- and imuthiol-treated animals with right neocortical lesions (these latter two groups did not differ significantly).

There may be a brain–liver–T cell pathway, but the target lymphocyte population(s) and the role of hemispheric lateralization of function need further replication and clarification. It would be interesting to vary the recovery period after surgery (ten weeks in the above experiments) and the site of the lesions. Ultimately, this should lead to a correlation with an anatomical difference between the hemispheres (e.g., differences in catecholamine tracts), and there should be an autonomic or neuroendocrine process that mediates the effect.

Early Studies of Conditional Immune Effects

Metalnikov and Chorine (1926, 1928) are generally credited with having conducted the first studies of conditional immune effects. They began by conditioning an increase in peritoneal leukocytes similar to that seen after an antigenic challenge. Guinea pigs were given daily treatments with a conditional stimulus (CS) (scratching or heating of the skin), followed by an intraperitoneal antigen injection (the unconditional stimulus (US): a small dose of tapioca, *Bacillus anthrax,* or a *Staphylococcus* filtrate) over a period ranging from 15 to 20 days. After a rest period of 12 to 15 days, the animals were given the CS only. The typical finding was an increase in peritoneal leukocytes similar in cell composition, but weaker and more transitory than those seen after an intraperitoneal injection of antigen. Data from three such conditioned animals and one unconditioned animal given an antigen were reported.

There followed three experiments that examined the survival of guinea pigs after a normally lethal injection of *Vibrio cholera* bacteria (Metalnikov and Chorine 1926). Conditioning was done essentially as before. A daily presentation of the CS, scratching, followed by an injection of the US, *Staphylococcus* filtrate (Experiment 5) or *B anthrax* (Experiments 6 and 7). The number of training trials varied among the experiments; 12 in Experiment 5, 25 in Experiment 6, and 18 in Experiment 7. Rest intervals between training and testing were given in two of the experiments (ten days in Experiment 5 and 15 days in Experiment 6). In each experiment, there were two conditioned animals and one or two control animals, which received neither the CS nor the US in training. In test trials in Experiments 5 and 7, only the conditioned animals were given the CS. On the following day, all animals were given an injection of *V cholera*. Only the conditioned animals survived. In Experiment 6, one of the conditioned animals did not receive the CS before the *V cholera* inoculation. The unstimulated (conditioned) animal died after 6 hours, the unstimulated control animals died after 7 or 8 hours, and the stimulated (conditioned) animal died after 36 hours. The conditioned survivors of Experiment 7 were also retested a month later, with only one receiving the CS before an injection of *Streptococcus*. This individual survived, whereas the unstimulated but conditioned animal and two controls died.

In their study of conditional antibody formation, Metalnikov and Chorine (1928) gave three rabbits 12 to 15 pairings of a CS (heating the ear or scratching the flank) and a US (an injection of heat-killed *V cholera*) over a period of two weeks. Three weeks after the last injection, blood samples were collected from all of the animals, and on the following day two were given the CS three times in 24 hours, while the other animal was left untreated. In the stimulated animals, the antibody levels (titers) rose slightly, reaching a maximum five to six days later. The titer in the unstimulated animal remained constant. Two subsequent experiments replicated this, with the only major change in procedure being the use of a trumpet blare for two to three minutes as the CS in the final experiment!

Considerable attention was given to the question of conditioned immune effects by Soviet investigators, first in the 1930s, and again in the 1950s and early 1960s. This work has been exten-

sively reviewed elsewhere (Ader 1981, Korneva, Klimenko, and Shkhinek 1978, Luk'ianenko 1961). Many of the experiments were basically similar to those done by Metalnikov and Chorine (1926, 1928), and apparently there was some controversy over the reproducibility of some experiments. The discussion here will focus on their assumptions about how Pavlovian conditioning should be studied and about neural control over immunity.

When Pavlov studied conditioned salivary responses in dogs, it was sufficient to note that before conditioning, a dog would not salivate after the sound of a bell, but that after conditioning it would do so. All that was needed for a control was this before–after comparison or another unconditioned dog. The emphasis was on observing conditioning in the individual animal, and it was noted that some animals conditioned better than others. The more typical approach today is to make group comparisons. Using the control procedures recommended by Rescorla (1967), one might compare the performance of groups in training given excitatory conditioning (e.g., the CS always followed by the US), noncontingent training (in which the presentation of the CS is random with respect to the presentation of the US), and inhibitory conditioning (in which the CS and US are explicitly unpaired), after all receive a CS-only test trial. This approach offers the advantage of controlling for psychological habituation and sensitization, but takes the emphasis away from the individual animal.

At the time of the early Soviet experiments, there was uncertainty as to how the specificity of the immune system was encoded and controlled. One hypothesis that aroused great interest among these scientists was that the nervous system somehow encoded the specificity. This seemed plausible because the nervous system is anatomically complex, and the mechanisms by which it supports complex functions were, and still are, poorly understood. Another influential theory suggested that *de novo* synthesis of an antibody could occur in conditional immune responses.

With these assumptions, it was not unreasonable to test for conditional immune effects by giving a small number of animals excitatory conditional training and testing their response to a CS-only trial. However, modern immunologic theory certainly has buried the concept that the nervous system encodes the specificity of immunity, and offers little support for the idea of *de novo* synthesis of antibodies in the absence of antigen. From what is now known about autonomic and neuroendocrine modulation of immunity, it is conceivable that an already active immune process might be enhanced or suppressed by a conditional change in autonomic or neuroendocrine activity. If *de novo* synthesis were to occur, it would be impossible to regulate its specificity; the animal would begin to make either all of the antibodies for which it had immunological memory cells, or, if a preceding primary immunological response were not required, all of the antibodies within the animal's extensive repertoire. Not only would this be energetically wasteful, presumably it would be inhibited, as are responses in antigenic competition. Indeed, presence of the antigen is generally considered to be a requirement for the initiation of antibody synthesis, and removal of the antigen is one of the ways in which antibody synthesis may be terminated. It is very unlikely that *de novo* synthesis of antibodies is made possible by Pavlovian conditioning.

Dealing with the immune system poses several further challenges for the experimenter attempting to demonstrate conditioning. The immune system itself shows "memory" independent of the nervous system. However, this also means that the experimenter must not only control for psychological habituation and sensitization, but also for immunologic sensitization (and habituation, which in immunology is termed tolerance, and can be induced in a variety of ways that directly involve lymphocytes and are independent of the nervous system). The early Soviet studies of classically conditional immune effects generally lacked such controls.

Brief mention should be made here of experiments done by Dolin, Krylov, Luk'ianenko, and Flerov (1960), because they were different in style from the others. They found that if animals were injected with saline during the course of repeated vaccinations, they would respond to a subsequent injection of the same antigen with a reduced level of antibody production, in comparison to animals given the same immunologic treatments without saline injections. In other experiments, animals were given an injection every four days, alternating between saline and an antigen. As a test, they were given the antigen at a time when the saline would normally be given. The result was a decreased titer when compared to trials in which the antigen was administered on its proper time in the cycle of alternating injections. This procedure was extended to the study of anaphylactic shock, with similar findings.

Recent Studies of Conditional Immune Effects

In the past ten years, a number of studies have reported conditional immunosuppression using a taste-aversion learning paradigm (Ader and

Cohen 1975, 1982; Ader, Cohen, and Bovbjerg 1982; Ader, Cohen, and Grota 1979; Bovbjerg. Ader, and Cohen 1982, 1984, Cohen, Ader, Green, and Bovbjerg 1979, Gorczynski and Kennedy 1984, Gorczynski, McRae, and Kennedy 1983, Klosterhalfen and Klosterhalfen 1983, Kusnecov, Sivyer, King, Husband, Cripps, and Clancy, 1983, Rogers, Reich, Strom, and Carpenter 1976, Wayner, Flannery, and Singer 1978). In these experiments, a novel taste, usually saccharin (CS), and a drug (*e.g.*, cyclophosphamide) (US), which supresses immunologic responses and engenders subsequent taste aversion, were jointly administered to experimental animals. Following one such training trial, it was possible to observe an impaired immune response to an antigenic challenge (and taste aversion) when saccharin again was added to the drinking water. This procedure could have direct clinical applications in the treatment of autoimmune disorders, and has been successfully applied to antibody production, graft–*vs.*–host disease, systemic lupus erythematosus, adjuvant arthritis, and natural killer cell activity (all in rats or mice). The only response in the literature that has not yielded statistically significant conditional immunosuppression is antibody production against *Brucella abortis* (Wayner, Flannery, and Singer 1978). This was attributed to *B abortis* being a T cell independent antigen; but Cohen, Ader, Green, and Bovbjerg (1979) obtained significant results with another T cell independent antigen.

As for the possibility that differences in fluid consumption between the experimental groups might be important, Ader, Cohen, and Bovbjerg (1982) controlled for this and still found conditional immunosuppression.

Recent studies by Gorczynski and his colleagues (Gorczynski and Kennedy 1984, Gorczynski, McRae, and Kennedy 1983) have shown that the time of day during which the test trial is administered influences the results of taste-aversion/conditional immune effects experiments. When the test trial was given early in the day the result was conditional immunosuppression. Testing at midday sometimes failed to produce significant results, and testing in the evening sometimes produced significant immune enhancement! (All training was done at midday.) The possibility of circadian rhythms in immune responses has not been extensively studied. However, Shifrine and Rosenblatt (1984) have observed that there are seasonal changes in immunity.

Gorczynski, MacRae, and Kennedy (1983) reported a correlation between the activity of mice in an "open field," and their subsequent conditional immune response in the taste-aversion model. Conditioned mice that had the lowest number of antibody-producing cells (IgM plaque-forming cells from spleen samples collected six days after immunization) had a lower activity in the open field (relative to other mice tested) before any of the conditioning training trials were done. Individual correlations between activity and (conditional) immune responses were high only among these animals; correlations were much lower for the conditioned animals with higher numbers of antibody-producing cells, conditioned animals not given the CS (saccharin) in the test trial, or mice given water plus cyclophosphamide during the training trials, and saccharin in the test trial.

It is interesting to note that although conditional immunosuppression of antibody production in rats can be obtained after just one training trial, Gorczynski *et al.* (1983, 1984) needed three training trials to produce conditional immunosuppression of the number of antibody-producing cells in mice. This could reflect a species difference, or it could represent a difference between effects due to the amount of antibodies being produced by individual cells, and effects due to the number of cells producing antibodies. No published conditioning experiments have compared serum antibody titers and the number of antibody-producing cells.

Gorczynski *et al.* have also produced conditional immune effects by means other than the taste aversion paradigm (Gorczynski, McRae, and Kennedy 1982). Allogenic skin grafts (CS + US) were repeatedly applied at 40-day intervals to mice. Some of these conditioned mice showed greater numbers of peripheral cytotoxic T lymphocyte precursors, specific for the alloantigens on the grafted tissue 12 days after a sham graft (CS alone) than did mice who in training had received sham grafts only (CS alone), injections of allogenic cells (US alone), or injections of allogenic cells followed by sham grafts (backwards conditioning, or possibly explicitly unpaired CS + US). Extinction by repeatedly applying the CS alone (sham grafts) abolished the conditional increase in peripheral cytotoxic T lymphocytes.

Smith and McDaniel (1983) conducted a conditioning study involving the human delayed hypersensitivity reaction to tuberculin. Nine healthy human volunteers (tuberculin positive) were given five monthly tuberculin skin tests to each arm as training. One arm always received tuberculin, the other saline. Subjects could clearly see that the solution applied to the right arm always came from a red vial and that the solution for the left arm always came from a green vial. One month after the training period the contents were switched without the knowledge of

either the subject or of the nurse administering the injections. No reactions due to any of the saline injections were seen during the experiment. The test trial responses to tuberculin (as measured by a nurse) were significantly diminished. One month later the subjects were retested with tuberculin again on the same arm in which tuberculin was given during the test trial (previously, during training, this arm had received saline). This time, however, the subjects were fully informed of the design of the experiment. Skin reactions were larger than on the previous test trial and closer to the values recorded during training on the other arm. This protocol is similar to biofeedback.

Physiologic Mechanisms of Conditioned Immune Effects

What might be the physiologic mechanisms that underlie these conditioned immune effects? In the taste-aversion model, a probable mechanism is an increased release of ACTH, resulting in immunosuppressive levels of corticosteroids. This is supported by the finding that taste-aversion learning with cyclophosphamide does produce increases in serum corticosterone levels (Ader 1976), and that adrenalectomy blocks such conditional immunosuppression (Gorczynski *et al.* 1983). However, taste aversion induced with lithium chloride does produce changes in serum corticosterone levels, but does not produce a statistically significant conditional immune effect. Ader, Cohen, and Grota (1979) tried to mimic conditional immunosuppression by giving lithium chloride or intraperitoneal injections of corticosterone to rats given the normal taste-aversion training with cyclophosphamide, and their regular drinking water on the test trial. These rats did not show the immunosuppression of their counterparts given normal training, and presented again with the conditional stimulus (saccharin in the drinking water; in fact, they did not differ significantly from the control rats who were given the same training and then only given water to drink on the test trial. A conditional release of ACTH does not easily explain the conditional immune enhancement observed by Gorczynski *et al.* (1983, 1984), in which testing was done in the evening rather than the morning. Apparently no one has measured both ACTH/serum corticosteroid levels and immunosuppression in a single experiment.

One possibility may be that, regardless of what the conditional physiologic response is (*e.g.*, changes in autonomic activity, or serum levels of ACTH or endorphins), there may be a requirement for an agent that strongly affects immunity during training. This has been the case for all recent and successful published reports of conditional immune effects. The list of USs used in the taste-aversion model now includes cyclophosphamide, antilymphocyte sera, acute (but not chronic) stress (Gorczynki *et al.* 1983, 1984), and polycytidylic:polyinosinic acid (which affects natural killer cell activity) (Gorczynski *et al* 1984). The control procedure for the direct influence of such agents has usually been to give two groups of animals the same conditioning training, then give only one the CS during the test trial. The control group is "conditioned," but does not give the conditional response (CR). Another control group is often given the immunoactive agent in training as well, but it first encounters the CS in the test trial (it is not conditioned).

Gorczynski *et al.* (1984) have suggested that the conditional increase in cytotoxic T lymphocyte precursors in their skin graft experiments may be the result of an increase in "lymphocyte trafficking": populations of lymphocytes in the bone marrow or other lymphoid organs are put into circulation. Cohen and Crnic (1984) have found that stress or glucocorticoid treatment results in the sequestering of mouse T cells in the bone marrow, and that the extent of sequestration correlates positively with the open field activity of the mice. This explains why an increase in cells specifically cytotoxic for the antigen used in training, but not for a new antigen added during testing, is seen; presumably, only the former has a large pool of cells in storage prepared against it. This mechanism is also compatible with the observation that the conditional increase in cells in the peripheral bloodstream is seen after a CS-only test trial. Autonomic innervation of the bone marrow and other lymphoid organs may be the mediator of this response; if so, one would expect that destruction of the sympathetic nervous system by means of six-hydroxydopamine would block it. Apparently, this has not been tested yet. Dann, Washtel, and Rubin (1979) found that tuberal hypothalamic lesions in rats stimulated allograft reactivity, and that hypophysectomies before the lesions did not block this, suggesting a direct neural pathway involved in transplantation immunology.

In addition, there are physiologic mechanisms that, although not directly involved in immune activities such as antibody production, mediate the effects of immune-related disorders and may be subject to Pavlovian conditioning. Dekker, Pelser, and Groen (1957) and Ottenberg, Stein, Lewis, and Hamilton (1958) demonstrated learned effects in asthma, and recently Russell, Dark, Cummins, Ellman, Callaway, and Peeke (1984) reported conditional histamine release. Such

mechanisms may at least in part be responsible for the conditional delayed hypersensitivity effect found by Smith and McDaniel (1983). Similarly, nonspecific bodily defenses (often given little attention in immunology) may be conditioned.

Conditioned Immune Effects: Meaning and Occurrence

Conceptualizing Pavlovian conditioning as a mechanism by which an organism can anticipate the onset of a biologically important event (the US), and initiate preparatory CRs to allow the organism to better deal with the US effects (Hollis 1982), invites the hypothesis that one reason neural control of immunity exists is to accommodate the adapative value of classical conditioning. In its natural environment, an animal with a cut or a scratch must mount an immunological defense against microorganisms. In the laboratory or a clinical setting, an antigen is reliably preceded by an injection. Therefore, conditional immune effects may in fact be very common. The difficulty for the investigator may not be inducing such responses (for a significant survival advantage, they should develop after only a few CS + US pairings), but employing the proper controls, both immunologic and psychological, to demonstrate that they exist.

It has been noticed that the conditional immunosuppression associated with the taste-aversion model is much smaller than the suppression usually observed with the immunosuppressive agent used as an US (Ader and Cohen 1975). This is not surprising given the probable physiologic basis of the conditional effects: an already occurring immune response is either enhanced or suppressed, or a response of intermediate probability may be made more or less likely to commence. In these cases, the conditional effect is really a secondary influence on an immunological function, which is dependent on conventional immunologic variables (such as the need for an antigen for the response to be initiated, or target cells for the response to be detected). This does not mean, however, that conditional immune effects are not important. A very small increase in the potential of the immune system might be of great survival value against pathogens, but also increase the occurrence and severity of allergies and autoimmune disorders. In this regard, it is worth keeping in mind that the immune system is composed of a variety of cells with different class receptors and functions; therefore, what may affect the antibody-producing cell may not affect the natural killer cell.

The taste-aversion model of conditioned immunosuppression has been used extensively and has produced some interesting results, but whether anything like it normally occurs is another matter. An animal might consume a toxin that engenders taste aversion and affects its immunity, but on subsequent encounters the taste aversion itself should be sufficient to prevent further consumption. Recall that taste aversion itself, such as when lithium chloride is used, apparently does not produce a conditional immune effect. A further conceptual problem is that the US in taste aversion can be considered to be stressful; thus, the CR may be a stress response. It is well-accepted that stress can impair immunity, and whether the stress is direct or conditioned probably does not matter. However, the research on autonomic and neuroendocrine modulation of immunity implies mechanisms that go far beyond the traditional concept of stress.

There is a need for more experiments that use USs that affect immunity, but are not themselves aversive or stressful. In this way, the conditional response is the regulatory response to an afferent signal from the immune system, not a stress response. It should be sufficient to use a CS that is aversive. Indeed, a skin irritant may be appropriate for a conditional immune effect in the same fashion that a novel taste is a salient CS for the sensations produced by lithium chloride injections. Such experiments would shed more light on the role of Pavlovian conditioning in immunity, and be a useful tool in further studying autonomic and neuroendocrine modulation of immunity.

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