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Methods Currently Used in the Pharmaceutical Industry for Evaluating Immunotoxic Effects

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I. Introduction

IMMUNOTOXICOLOGY provides an important additional dimension in assessing the safety of drugs. Numerous studies have been done with a variety of methods that measure different aspects of immune competence, for a review, see Koller (1, 2) and Vos (5). The purpose of this paper is to summarize the kinds of methods that are used to evaluate potential immunotoxic effects of drugs in experimental animals determined by a survey of specific toxicology laboratories in the pharmaceutical industry.

II. A Survey Approach

A questionnaire was prepared to gather data on current industry practices and mailed to 80 companies, most of whom are members of the Drug Safety Subsection (DRUSAFE) of the Pharmaceutical Manufacturers Association. The survey was conducted in a way that maintained confidentiality. Those responding to the questionnaire were asked to check all methods that their particular firm had performed. Realizing that many pharmaceutical companies are engaged in considerable activity related to immunoassay or to the immunological aspects of drug efficacy and/or safety, the objective of this questionnaire was to determine the immunological parameters used in toxicity and safety evaluation. While two questions were concerned with when immunocompetence was evaluated and the species in which immunotoxicity studies were conducted, all remaining inquiries concerned the kinds of assays that were performed as part of immunotoxicity testing (see table 1). In all cases, allowances were made for replies other than those indicated in the questionnaire.

III. Results and Discussion

Eighty surveys were distributed and a total of 45 responses (56%) were received. Nineteen of the 45 responders (42%) indicated that their firm had not conducted immunotoxicity studies. The responses from the remaining 26 companies were then analyzed from the following perspectives.

A. Stage of Evaluation (Fig. 1)

The majority of firms (17, or 65%) evaluate immunocompetence during the preclinical stage of toxicity assessment, but it is not clear from this survey how often immunotoxicity studies are performed and whether all compounds or just a select few are tested. Fourteen laboratories (54%) conduct immunotoxicological tests as part of "specialty studies," inferring that immune function tests are only performed as a follow-up to routine preclinical or clinical studies, at the request of regulatory agencies, or for registration purposes only. It was also noted that one company had conducted immunotoxicity studies during clinical evaluation.

B. Species Used in Immunotoxicity Studies (Fig. 1)

Dogs (62%), rats (54%), and guinea pigs (54%) are the most commonly used species in immunotoxicology, followed by the mouse, rabbit, and monkey. The majority of companies (77%) use more than one species.

C. Hematological and Histopathological Examinations (Fig. 2)

Nearly all firms conduct routine hematological (22, or 85%) and histopathological (23, or 88%) examinations. Of those responding to the survey, 81% (21) indicated that both parameters were examined simultaneously, whereas two organizations apparently did not perform either hematology or histology of lymphoid organs.

D. Contact Allergenicity (Fig. 3)

One important indication of a toxic effect is whether a given drug can induce an allergic reaction manifested by skin sensitization. Two popular methods, including the classical Landsteiner-Draize test (3) and the Magnusson-Kligman maximization assay (4), measure the ability of chemicals to elicit a delayed-type hypersensitivity reaction on the skin of guinea pigs. More than half (15, or 58%) of the institutions conduct allergenicity testing. Ten companies (38%) perform the Landsteiner-Draize test, while nine firms (35%) conduct maximization assays as part of their immunotoxicity studies. Both methods appear to be used with equal frequency, despite the fact that the maximization test is considered to be more sensitive and predictive of human risk than the Landsteiner-Draize test (4). Four of the companies that responded did both tests for contact sensitization.

TABLE 1
Kinds of assays that could be used in immunotoxicity testing of drugs

General Area	Tests
Hematology	Total leukocyte count, differential
Histopathology	Spleen, thymus, bone marrow, lymph node, Peyer's patches, fluorescence immunopathology
Contact allergenicity	Landsteiner-Draize, maximization
Serum biochemistry	IgG, IgM, IgA, IgE
Antibody response	Plaque assay, Mishell-Dutton assay, hemagglutination, radioimmunoassay, enzyme-linked immunosorbent assay, viral infection, bacterial infection
Blastogenesis	Phytohemagglutinin, concanavalin A, lipopolysaccharide, pokeweed mitogen, specific proteins, mixed-lymphocyte culture
Cell-mediated immunity	Graft-versus-host reaction, graft rejection, delayed hypersensitivity, viral infection, bacterial infection, tumor immunity
Mediator production	Interferon, migration inhibition factor, macrophage activation factor
Reticuloendothelial system	Clearance in vivo, phagocytosis in vitro, cytostasis/cytotoxicity, chemotaxis, migration inhibition, enzymatic activation
Enumeration	Cytolysis, rosetting, cell sorting, flow cytometry, immunofluorescence, histochemical, electrophoresis
Host resistance models	Viral, bacterial, fungal, parasitic, malignancy
Stem cells	Colony formation, cell sorting, cell transfer studies
Autoimmunity	Antinuclear antibodies, circulating antibodies, immune complexes
Polymorphonuclear leukocytes	Immediate hypersensitivity, degranulation, chemotaxis, phagocytosis
Miscellaneous	Complement levels/activity

E. Serum Biochemical Examinations (Fig. 4)

Nine firms (35%) analyze serum gamma globulin fractions, either by electrophoresis (three) or by determining serum immunoglobulin levels, such as IgG (six), IgM (three), IgA (three), and IgE (four). All six institutions that measure immunoglobulin levels examine more than one immunoglobulin with IgG being universally determined. One company also measures steroid concentrations, which is useful in determining whether a particular drug-induced immunosuppression might be secondary to a stress-related increase in adrenal cortical activity.

F. Humoral Immunity (HI) (Fig. 4)

While measurements of serum globulins by quantitative assays may indicate an altered immune response, an observation such as this is superficial because it is necessary to determine by (usually more sensitive) immunological methods effects on specific aspects of the immune response (1). The immune response to an antigen (i.e. pathogens and other foreign materials) characterized by the elaboration of specific immunoglobulins, called antibodies, is referred to as HI, simply because antibodies can be found in the serum, or fluid phase of the body. The cells that produce antibody are called plasma cells and are derived from lymphocyte precursors in the bone marrow (B cells).

Nine companies (35%) have examined HI function. The most common technique used is hemagglutination (six), which measures serum hemagglutinating antibody to sheep erythrocytes. Antibody responses to such antigens, known as T-dependent antigens (require T helper cells and macrophages), are perhaps the best example of a composite immune function test involving several cell types working cooperatively in a close interaction. More sensitive methods that measure serum antibody titers, such as the enzyme-linked immunosorbent assay and radioimmunoassay, are not in widespread use (less than 10%), probably because of their relatively recent development and/or level of sophistication. Only two laboratories attempt to determine antibody titers during bacterial infection. Finally, as a direct measurement of the number of antibody producing cells, five companies quantify plaque-forming cells in the spleen.

If serum immunoglobulin levels and the assay of antibody responsiveness are considered together, 15 of the 26 companies (58%) conducting immunotoxicity testing perform at least one test from either area. However, more than half (eight) of these 15 laboratories do only one test; six firms concentrate solely in the area of serum immunoglobulin determination, the less specific, less sensitive approach.

G. Cell-Mediated Immunity (CMI) (Fig. 5)

Assays of CMI measure the function of thymus-derived lymphocytes (T cells). In addition to T helper cells, there exist other subpopulations of T cells that mediate delayed hypersensitivity reactions, cytotoxic responses characteristic of graft rejection, bactericidal activity, the graft-versus-host reaction (T killer cells), and those that produce lymphokines, or mediators of a variety of immunological activities. Polyclonal stimulation of DNA synthesis (termed blastogenesis or transformation) by the mitogens concanavalin A and phytohemagglutinin are also useful in measuring T-cell function.

Thirteen companies (50%) conduct assays for CMI. Delayed-type hypersensitivity is the most popular method (nine), followed by blastogenesis (eight), the graft-versus-host reaction (five), and mediator production (four). Antibacterial immunity and graft rejection were only used by one company each. Blastogenesis induced by pokeweed mitogen (which stimulates B cells as well as T cells) and specific proteins are also used infrequently (one firm each). Of the four laboratories examining lymphokine activity, three analyze macrophage migration inhibition factor. Macrophage activation factor and interferon were also mentioned on one occasion each.

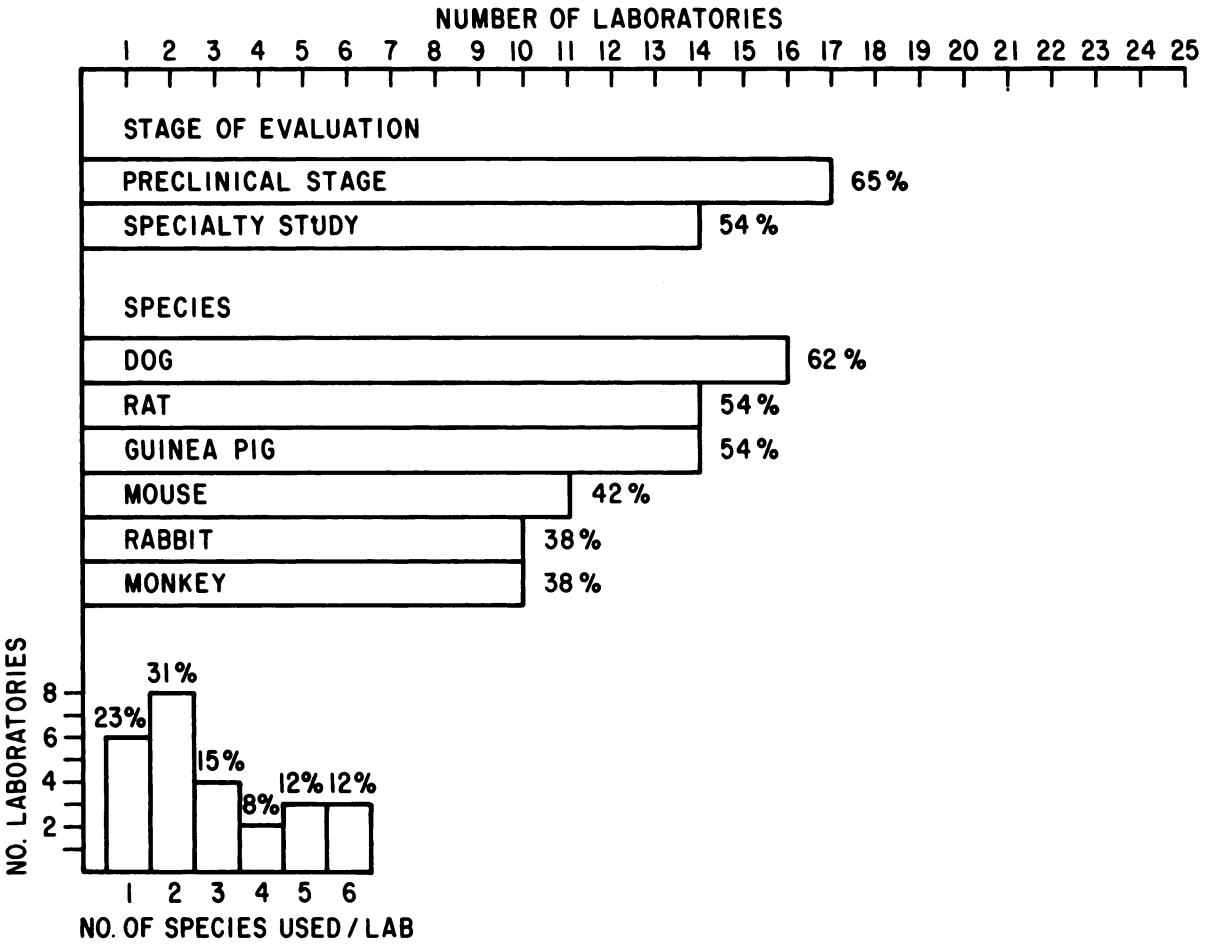

FIG. 1. Summary of data from 26 laboratories conducting immunotoxicity testing: Stage of evaluation and species.

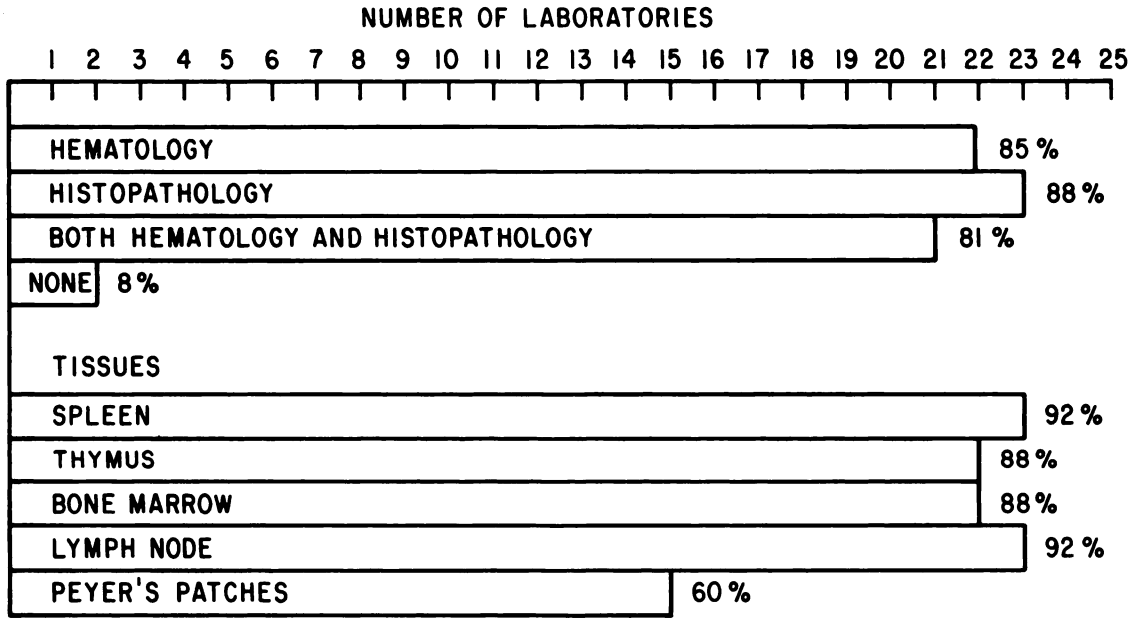

FIG. 2. Summary of data from 26 laboratories conducting immunotoxicity testing: Hematology and histopathology.

Most institutions seem to favor in vivo tests compared to in vitro tests alone. Only two companies perform the in vitro lymphoproliferative assay (blastogenesis) without supplementary in vivo tests. The acceptability of this latter approach remains to be validated since blastogenesis is recognized as a crude measure of clonal expansion by a stimulant of controversial biological significance.

Interestingly enough, the trend indicates that most

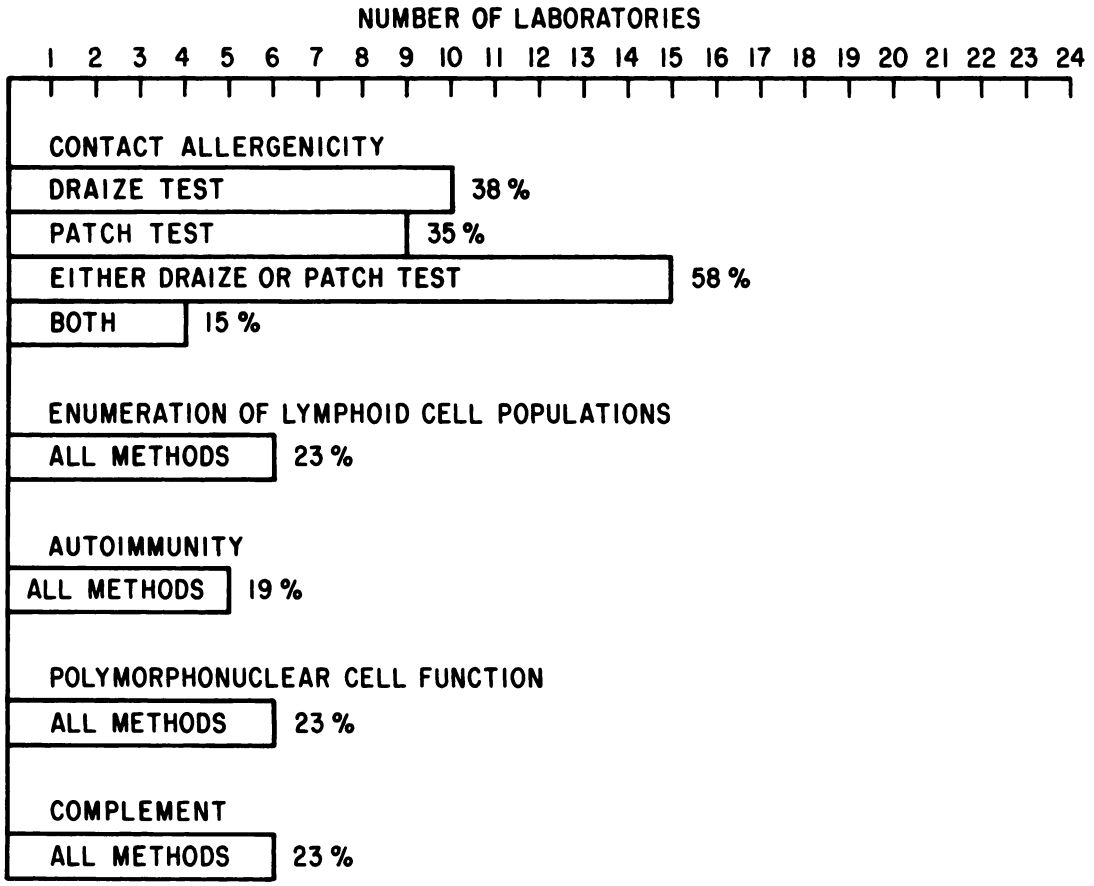

FIG. 3. Summary of data from 26 laboratories conducting immunotoxicity testing: Contact allergenicity, enumeration of lymphoid cell populations, autoimmunity, polymorphonuclear cell function, and complement.

companies prefer to conduct assays in both CMI immunity and HI (10 of 18 companies responding to inquiries in both areas). The majority of laboratories (eight of 13, or 62%) perform two or more tests.

H. Reticuloendothelial System (RES) (Fig. 5)

Eight companies (31%) responded positively to studying some functional aspects of the RES. Yet, no clear consensus emerged among the assays selected. Four laboratories perform chemotaxis assays, whereas in vitro phagocytosis (three), in vivo clearance (two), and cytostatis/cytotoxicity tests (two) are less frequently done.

If all tests listed under HI (including serum immunoglobulin levels), CMI, and RES are considered as representative of an immune function profile, then 19 of 26 companies (73%) that responded affirmatively to conducting immunotoxicity studies perform at least one test from one of these three broad areas. In contrast, only four laboratories indicated that they perform a more comprehensive survey, which includes a minimum of one test from each of the three areas. Of the 26 firms that perform immunotoxicity studies, seven do not conduct any immune function test listed as HI, CMI, or RES.

I. Enumeration (Fig. 3)

Quantifying lymphocyte subpopulations in experimental animals can be tedious and time-consuming. Whereas certain reagents, such as monoclonal antibodies, are available for a limited number of species, most methods are not mechanized and tend to be either subjective (e.g., rosetting, immunofluorescence, and histochemical techniques) or not conducive to routine analyses (e.g., flow cytometry and electrophoresis). Fluorescence-activated cell sorting is such a novel technique, in addition to being cost-prohibitive, that few laboratories (only one in our survey) have made the transition to this system.

Only six companies (23%) attempt to enumerate lymphocyte subpopulations beyond mere hematological examination. Once again, no one method has gained uniform acceptance among the various laboratories: Two each for electrophoresis, immunoflorescence, and the rosette test, and only one performing a histochemical assay.

J. Autoimmunity (Fig. 3)

Nineteen percent (five) of the firms use methods that measure autoimmunity, specifically, immune complex formation in tissue (three) or determination of antibodies against autologous DNA or other cellular components (two). Autoimmune reactions may reflect drug-induced alterations of normal cells (either by direct binding or modification of the cell surface), which trigger the immune response against "self" antigens. On the other hand, autoimmunity related to drug-treatment may sug-

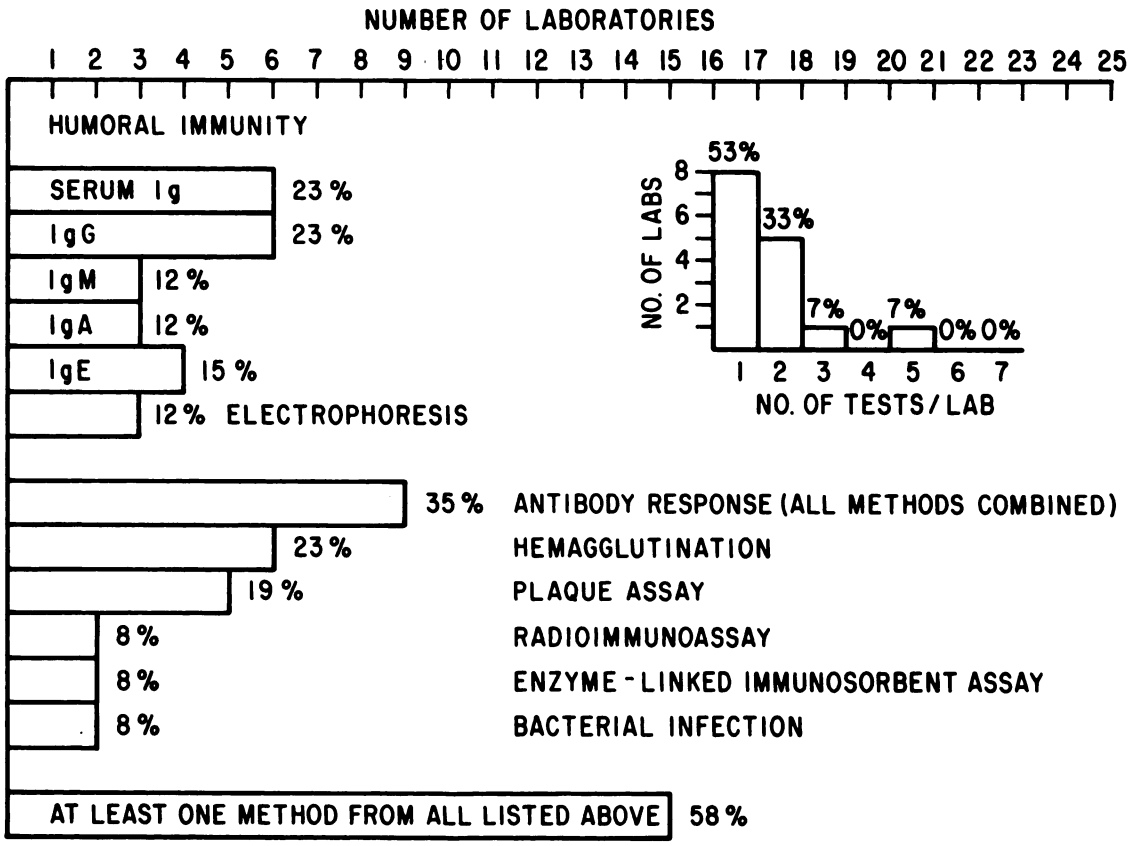

FIG. 4. Summary of data from 26 laboratories conducting immunotoxicity testing: Humoral immunity.

gest an alteration of the complex immunoregulatory mechanism that prevents the immune system from reacting out of control.

K. Polymorphonuclear Leukocytes (PMNs) (Fig. 3)

Although not involved in the immune responses cited above, PMNs comprise a significant portion of the circulating white cells and are involved in the acute and early phases of all inflammatory responses, referred to as immediate hypersensitivity. Most cells, or fixed basophils, are initiated by airborn antigens, such as pollen, to release histamine (a degranulation response). Neutrophils can move either in the direction of certain stimuli, called chemotaxis, or phagocytize and kill bacteria. Only six companies (23%) of those polled perform assays in this area. Five of the six laboratories study immediate hypersensitivity, whereas degranulation (two), chemotaxis (two), and phagocytosis (one) were cited less frequently.

L. Complement (C) (Fig. 3)

Complement refers to a series of proteins that mediate a variety of reactions depending on the stage of activation, most notably chemotaxis and membrane dissolution or damage. Six (23%) firms measure either C activity or serum C levels.

IV. Conclusion

No survey can provide all the information necessary to make universal statements since it is difficult to achieve a 100% return from the original distribution and there is always the possibility that some organizations were inadvertently omitted. It does appear, however, that a significant number (26 of 45, or 58%) of companies (19 not conducting immunotoxicity studies plus seven not performing any immune function test) would be unprepared to answer immunologically related questions posed by regulatory agencies concerning new product candidates. It is not clear whether any of the 19 firms that responded negatively to immunotoxicity testing are in fact subcontracting this work elsewhere.

The results of this questionnaire demonstrate no consensus as far as the kinds of immunotoxicological assays being conducted by many laboratories throughout the country. However, in an effort to summarize the data, a typical screen has been constructed for evaluating immunotoxic effects based on responses of greater than 30% in any given area or greater than 40% for any given test within a given area (see table 2).

The general areas listed in table 1 (proposed tests) that were most notably absent from table 2 (most preferred tests) are the host resistance models, stem-cell function, and polymorphonuclear leukocyte activity. While some host resistance models may represent a composite immune response that is more relevant to human infectious disease, this approach must be managed carefully so that the integrity of the toxicity study is not jeopardized and the risk to other studies within the facilities is minimized. Stem-cell analysis has recently been heralded as a useful predictor of toxicity in the early stages of immune cell

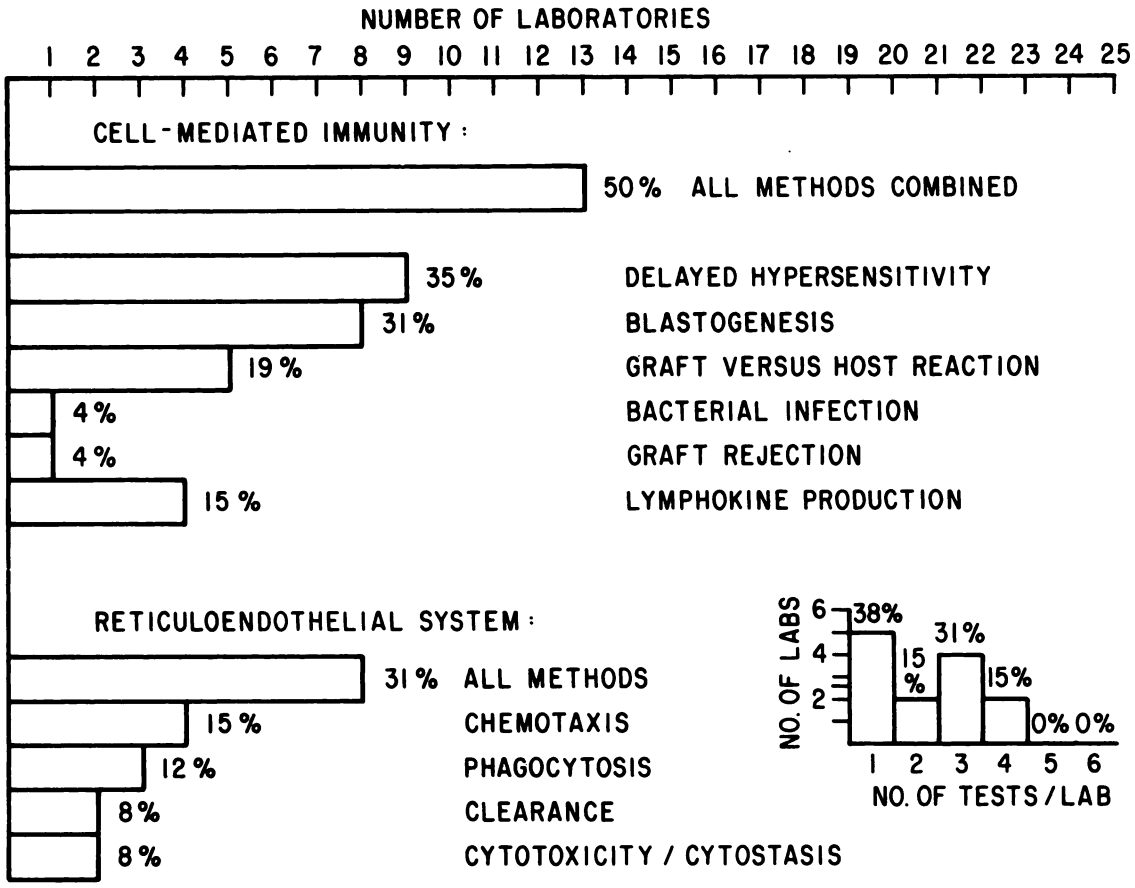

FIG. 5. Summary of data from 26 laboratories conducting immunotoxicity testing: Cell-mediated immunity and reticuloendothelial system.

TABLE 2

Typical screen for immunotoxicity testing representative of the PMAF survey

General Area	Tests
Hematology	Total leukocyte count, differential
Histopathology	Spleen, thymus, bone marrow, lymph node, Peyer's patches
Contact allergenicity	Landsteiner-Draize, maximization
Serum biochemistry	IgG, IgE
Antibody response	Hemagglutination, plaque assay
Blastogenesis	Phytohemagglutinin, concanavalin A
Cell-mediated immunity	Delayed hypersensitivity, graft-versus-host reaction
Reticuloendothelial system	Chemotaxis

maturation. However, such assays are currently performed by only a few laboratories in the country (not included in this survey).

Immunotoxicology appears to be gaining more widespread attention in the pharmaceutical industry. The fundamental aspect of its growth is the acceptance of the need to incorporate specific immune function tests into the basic toxicity study allowing correlation with other toxicological parameters, such as hematology and pathology. If the areas of serum biochemistry, cell-mediated immunity, blastogenesis, antibody response, mediator production, and reticuloendothelial system are considered together as typical immune function parameters, this survey showed that 73% (19 of 26) companies responding perform at least one of these tests. However, seven of these 19 firms now study one area alone, which seems insufficient because of the complexity of the immune system (viz., different cell types and variety of mechanisms involved in the immune response). None of the seven laboratories evaluate antibody response, the more composite immune function test.

The scope of immunotoxicological evaluation, however, is not limited to effects on immune function. The ability of a drug to elicit an allergic reaction is a common test that has existed for over four decades and is required by many regulatory agencies. Another area of importance is the induction of autoimmunity by chemically modifying the surface of autologous cells, resulting in an immunological reaction against normal tissue. Whether immunopotentiating compounds will increase the likelihood of autoimmune disease will also need to be addressed in the future.

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