

MODULATION OF IMMUNITY BY MACROPHAGES

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1. INTRODUCTION

The literature concerning the effects of macrophages on the immune response is already large and growing rapidly. Macrophages seem to be involved in almost every phase of the immune response. Not only are they important as effector cells in their own right, but there is now a wealth of information suggesting macrophages may, through a combination of suppressing and enhancing effects, play an important role in regulating the immune response. Evidence obtained both *in vivo* and *in vitro* supports the concept of macrophages as immunoregulatory cells. We shall review here the available *in vivo* and *in vitro* evidence for augmentation and suppression of immune response by macrophages, and will also suggest how macrophages may play a role as effector cells in T cell suppression and antigenic competition.

Many of the *in vitro* experiments, on which important conclusions have been based, have compared the results obtained in assay systems using cell populations depleted of macrophages to those obtained using cell populations containing, or highly enriched for, macrophages. Before discussing the details of these experiments, it is important to note that there are problems with interpreting such data.

There is virtually no way to prepare cell populations completely free of macrophages. None of the available methods have been shown to reduce the proportion of macrophages below 0.1 per cent, and this may not be sufficient to rule out the role of very small numbers of macrophages as auxiliary cells for the induction of immune responses. Most of the macrophage depletion procedures are based on adherent or phagocytic properties of macrophages. Petri dish adherence (Mosier, 1967) allows separation of the cells into two populations which can be tested alone or after recombination. The resultant populations, however, are by no means pure and the degree of depletion of macrophages is not as good as that achieved by other techniques. Passage of lymphoid cells over adherence columns of nylon wool (Julius *et al.*, 1973), rayon cotton (Kirchner *et al.*, 1974b), glass beads (Shortman, 1968), or Sephadex G-10 (Ly and Mishell, 1974) allows rather good depletion of adherent cells. However, since B cells are adherent, albeit less than macrophages, those procedures most effective in depleting adherent cells must of necessity deplete a certain proportion of the B cells. In fact, a procedure based on this principle has been described to obtain a purified population of T cells simply by depleting all of the adherent cells (Julius *et al.*, 1973). Since, however, as many as 80 per cent of the cells are lost when performing such procedures, it is obvious that even some T cells are depleted. In addition to the separation procedures based on the adherent properties of macrophages, incubation of cells with powdered iron to allow phagocytic cells to ingest the iron particles, and then passage of the cells through a strong magnetic field to obtain a population free of phagocytes (iron and magnet technique), has been a useful technique to obtain a phagocyte-depleted population (Kirchner *et al.*, 1974b). However, since the percentage of the cells recovered (50-60 per cent of normal spleen cells) is routinely lower than the percentage of cells which are non-phagocytic, it is clear that even this depletion technique is not entirely selective for macrophages. Another approach to the problem of obtaining purified cell populations has been the

use of procedures wherein lymphocytes are allowed to rosette with heterologous erythrocytes and then placed over a Ficoll-Hypaque density gradient which separates the rosette-forming cells (T cells) from the non-rosette-forming cells. The use of such procedures has been reported in guinea pigs (Rosenstreich and Oppenheim, 1976) and humans (West *et al.*, 1976). Combinations of adherence depletion techniques and enrichment techniques such as just described seem likely to yield the purest populations. In addition to the above techniques, several substances and compounds have been reported to be selectively toxic for macrophages. Silica and carrageenan (Allison *et al.*, 1966) have been used *in vivo* and *in vitro* to deplete macrophage activity. Trypan blue (Hibbs, 1974) has been reported to be similarly effective. Finally, there are several reports of the use of 'specific' anti-macrophage sera and complement to kill macrophages (Feldmann and Palmer, 1971; Gorczynski, 1976). Unlike mouse T cells which express allelic forms of theta and Ly antigen on their surfaces, allelic forms of macrophage surface antigens have not been identified in any species thus far. Therefore, the antisera must be produced in heterologous species and attempts made to render them specific, by extensive absorption procedures with cell populations free of macrophages. However, as is apparent from the above discussion, macrophage-free populations are difficult to prepare, and further, if readily available, they would largely obviate the need for anti-macrophage sera. The difficult problems in this area have been reviewed by Leibovich and Ross (1975).

Preparation of *pure* macrophage populations is also difficult. In rodents and guinea pigs, a population enriched for macrophages can be obtained by washing out the peritoneal cavity, and many investigators refer to peritoneal exudate cells and macrophages interchangeably. Mice normally have $2-3 \times 10^6$ macrophages resident in the peritoneal cavity which can be washed out. It is important to note, however, that the resultant population is heterogeneous, consisting of about 60 per cent lymphocytes, 35 per cent macrophages, and occasional mast and other cells (Edelson and Cohn, 1976). If irritating agents such as light mineral oil, 10 per cent proteose peptone broth, or thioglycolate are placed in the peritoneal cavity, a sterile peritonitis develops. Initially there is an influx of neutrophils, maximally present about 48 hr after injection of the irritant. By 72-96 hr, most of the neutrophils have disappeared and macrophages make up the majority of the cells in the peritoneal exudate (PEC). Shevach (1976) reported that in guinea pigs, 3 days after *i.p.* injection of light mineral oil, the PEC consisted of 75 per cent macrophages, 10 per cent neutrophils and 15 per cent lymphocytes. By allowing such PEC to adhere to glass slides for 3 hr and washing off the nonadherent cells, a population consisting largely of macrophages and neutrophils remained (Lipsky and Rosenthal, 1973). Neutrophils, unlike macrophages, do not survive overnight incubation in media at 37° (Edelson and Cohn, 1976). Thus, Lipsky and Rosenthal (1973) reported that after overnight incubation, the adherent PEC remaining on the slides after vigorous washing were 99 per cent pure macrophages. Erb and Feldmann (1975a) used similar adherence techniques to purify PEC from mice. In addition, they treated the adherent PEC with both an anti-T cell and anti-B cell serum and complement followed by irradiation with 200 R. They reported that after thorough washing the macrophages were 99.7 per cent pure. Katz and Unanue (1973) used similar procedures in mice, injecting 1 ml of 10 per cent proteose peptone broth to induce the PEC. After 3 days, $8-15 \times 10^6$ PEC could be obtained which were 85 per cent pure macrophages. When such mice were irradiated with 660 R 24 hr prior to harvesting, only 4×10^6 PEC were recovered but 98 per cent were macrophages. In humans, PEC are obviously not readily available. However, Rode and Gordon (1974) could obtain essentially pure populations of monocytes from human peripheral blood leukocytes (PBL) separated from the red blood cells (RBC) with a Ficoll-Hypaque gradient, by allowing the PBL to adhere to glass Petri dishes for 8 days and vigorously washing off all remaining nonadherent cells. It should be noted that most of the procedures used to induce macrophages in the peritoneal cavity, or to isolate the macrophages, cause activation and other functional changes. For example, adherence to a surface results in increased protein synthesis and increased enzyme production by macrophages (Bodel *et al.*, 1977).

Whatever procedures are used to prepare macrophage-depleted or -enriched cell populations, it is obvious that the purity of the resultant population must be documented. Several methods are available for identification of macrophages and monocytes, but none are entirely satisfactory. The ingestion of various substances such as latex particles, colloidal carbon and heterologous erythrocytes has been used to identify phagocytic cells. In addition, histochemical staining techniques (Goldstein and Blomgren, 1973), particularly for nonspecific esterase (Yam *et al.*, 1971), are now widely used to identify macrophages. However, it is possible that even these methods may be fallible. Nathan *et al.* (1976) have expressed concern that a population of cells that they have isolated from the peritoneal exudates of mice pretreated with Bacille Calmette-Guerin (BCG) which have strong anti-proliferative effects on tumor cells *in vitro*, although adherent and staining positive for esterase, may be non- or poorly phagocytic under the conditions they used. They have been reluctant to call these cells macrophages and thus raise the question of whether cells other than macrophages and monocytes may stain positive for nonspecific esterase. Confirmatory reports of a discrete population of esterase positive, non-phagocytic cells, however, have not yet appeared. Enumeration of macrophages by any of the procedures is complicated by the fact that in any cell population there are not only mature macrophages and monocytes but also macrophage and monocyte precursors which may not be readily identifiable. These cells, although less adherent (Cline and Sumner, 1972), much less susceptible to anti-macrophage sera and complement (Virolainen *et al.*, 1972), and probably non-phagocytic, can mature during *in vitro* culture and function as macrophages even after the most efficient depletion procedures based on these functional properties. Many of these problems have been extensively discussed in a recent book (Bloom and David, 1976).

Macrophages appear to be a heterogeneous group of cells, both functionally and morphologically. At the present time it is not clear whether the heterogeneity is simply a result of different levels of maturation or whether there are in fact distinct subpopulations of macrophages which can be identified on the basis of certain cell surface or functional characteristics. During maturation from promonocyte through monocyte and immature macrophage to mature macrophage, there are many changes in their functional characteristics. Territo and Cline (1976) have provided evidence that the frequency of cell division decreases as the cells mature, while other characteristics such as phagocytic activity, glass adherence, microbicidal activity, surface receptors for immunoglobulin and the ability to interact with lymphoid cells in various immune reactions, generally increase.

Macrophages or monocytes from different sources display divergent characteristics. Bennet (1966) has shown that when macrophages from different tissue sources were isolated and cultured *in vitro*, their rate of attachment to glass as well as their mitotic rate in culture varied. Based on these features, Bennet described three categories of macrophages: peritoneal and peripheral blood macrophages that attached rapidly and had a low mitotic rate; bone marrow, spleen and liver macrophages that spread slowly and had a high mitotic rate; and alveolar macrophages that spread rapidly and had a high mitotic rate. Nitulescu and Förster (1976) reported that there were antigenic differences between the alveolar and peritoneal macrophages in rats. Walker (1967a) has also discussed the differences in functional activity between macrophages from various sources. He noted that peritoneal macrophages had a higher bactericidal activity than did alveolar macrophages and in addition that they had higher Fc receptor avidity. Furthermore, peritoneal macrophages responded well to chemotactic agents and were very efficient at rendering antigen immunogenic, while alveolar macrophages had low activity in these tests. Similarly, Boumsell and Meltzer (1976) have noted differences in the chemotactic response of blood monocytes and peritoneal macrophages to lymphocyte and complement-derived chemotactic substances with regard to both time-course and dose response. Hence, there is evidence that macrophages from different tissues may vary considerably in their functional characteristics. Whether this is due to stage of maturation or to subpopulation distribution is not clear.

Macrophages from a single tissue source may also be heterogeneous in terms of their surface characteristics and their functional reactivity. Rhodes (1975) found that there was marked variability in the avidity of the Fc receptors among guinea pig peritoneal macrophages. If the macrophages were induced with an inflammatory agent, there was a six-fold increase in the proportion of macrophages with high avidity Fc receptors. Heterogeneity in macrophage function has been noted by a number of investigators and has recently been reviewed by Walker (1976a). Basically, two types of observations suggested there may be functional subclasses of macrophages: (i) only a certain percentage of macrophages performed certain functions under similar conditions (such as phagocytosis of bacteria or erythrocytes), or (ii) macrophage populations which were fractionated based on their density or size, could be identified on the basis of various functional characteristics. Walker (1976b) used a discontinuous Ficoll gradient to fractionate rabbit peritoneal exudate cells into five subpopulations. Several different functional tests were then performed with the cells from each fraction. He found that binding of ^{125}I -T2 antigen was greatest in fraction 1 (least dense) while binding through the Fc receptor was greatest in the denser fractions 3 and 4. In addition, Walker fractionated rat peritoneal exudate cells from animals that had been immunized with a chemically induced tumor (plus BCG) and found that the cytotoxic reactivity of the macrophages resided predominately in two fractions. Similarly, differences in the size of macrophage effector cells were noted by Holden *et al.* (1976) in studies of the growth inhibitory activity of macrophages isolated from murine sarcoma virus-induced tumors. There were two functional subpopulations with different cell sizes (as determined by sedimentation velocity at 1 g).

All of these studies, however, are complicated by the fact that different methods of induction were used for harvesting macrophages and the cells may exhibit various levels of activation. In addition, the process of activation may call in a specific subpopulation of cells or, as suggested recently by Bomford and Olivotto (1975) and Woodruff *et al.* (1975), the activation process may cause macrophage precursors to differentiate into mature cells. Unfortunately at this time there is not enough information on subpopulations of macrophages from any one source to determine whether different functions will regularly be associated with particular macrophage subpopulations.

In all, it is apparent that dogmatic statements concerning the absolute requirement for macrophages, or the lack thereof for various immune responses, require careful documentation to be at all convincing. However, despite the above problems, it has still been possible to extensively study the role of macrophages in enhancing and suppressing immune responses, and evidence has been presented that macrophages play a vital role in regulating the immune response.

2. ENHANCEMENT OF THE IMMUNE RESPONSE BY MACROPHAGES

Macrophages have been shown to enhance almost every phase of the immune response. For many immune responses generated *in vitro*, there appears to be an absolute requirement for macrophages. This, of course, does not prove that macrophages are required *in vivo* or, if they are required, that they perform the same function *in vivo* as they do *in vitro*. When lymphocytes are cultured without macrophages, the viability after 4–5 days under some conditions is lower than when macrophages are present (Chen and Hirsch, 1972; Bevan *et al.*, 1974). In contrast, there is little or no evidence that macrophages enhance the viability of lymphocytes *in vivo*. Thus, it would seem that enhancing effects of macrophages on the immune response *in vitro*, which result *only* from the improvement of lymphocyte viability, may have limited relevance *in vivo*. One way to prevent such problems would be to perform *in vitro* investigations under optimal tissue culture conditions which are not substantially improved by the addition of macrophages. *In vivo* experiments on the

role of macrophages in enhancing immune responses also have problems. Observations made *in vivo* occasionally raise as many questions as they answer. Subclinical infections or fluctuations of hormone levels may affect *in vivo* experiments in ways that would be difficult to predict. However, with a combination of *in vivo* observations, and *in vitro* experiments designed to dissect and explain the results obtained *in vivo*, meaningful interpretations may be suggested. In this section we shall discuss the evidence that macrophages can enhance immune response *in vivo* and then we shall discuss the evidence obtained *in vitro* as possible explanations for the *in vivo* findings. Recent concepts regarding the mechanisms of the enhancing effects of macrophages, including some of the current concepts of antigen presentation, will be reviewed.

2.1. ENHANCEMENT OF THE IMMUNE RESPONSE IN VIVO

There is considerable evidence that macrophages can enhance the immune response *in vivo* (Mitchison, 1969; Klaus, 1974; for reviews see Unanue, 1972; Oppenheim and Seeger, 1976). This is supported by many experiments in which injection of soluble protein antigen bound to macrophages (by exposure to live macrophages *in vitro* or *in vivo*) has resulted in stronger immune responses in experimental animals than has injection of equivalent quantities of free soluble antigen. In addition, there is evidence that certain immunodeficient states are a result of an incompetent reticuloendothelial system (Carter and Rector, 1972; Hardy *et al.*, 1973; Blaese, 1975).

Both humoral and cellular responses have been shown to be enhanced by injection of antigens bound to macrophages (Unanue, 1972; Oppenheim and Seeger, 1976). Routinely, macrophages have been washed after exposure to radiolabeled soluble antigen and then injected into the host. The amount of antigen injected with the macrophages has been determined by the radioactivity of the injected cells. With some exceptions (to be discussed below), the response was ten- to a thousand-fold higher on a per microgram basis when the antigen was injected bound to macrophages (Mitchison, 1969; Klaus, 1974). Other cells such as fibroblasts can bind approximately the same amount of soluble antigen as macrophages, but there is general agreement that antigens injected bound to fibroblasts fail to augment strong immune responses (Mitchison, 1969; Klaus, 1974). As has been found with other macrophage functions, the ability to enhance immunogenicity is radioresistant (Unanue, 1972). However, viable macrophages are required. Mitchison (1969) has shown that antigen transferred on dead macrophages was no more immunogenic than antigen injected alone. Also, the ability of the transferred cells to interact with host cells is required. Macrophages in Millipore chambers implanted into the peritoneal cavities of mice did not enhance immunogenicity (reviewed by Unanue, 1972).

Although many soluble antigens are more immunogenic when transferred bound to macrophages, some are as immunogenic when injected alone. The ability of macrophage-bound antigen to enhance immunogenicity has been shown to be inversely related to the ability of macrophages to clear the antigen from the serum *in vivo* (reviewed by Unanue, 1972). Keyhole limpet hemocyanin (KLH) was taken up very well by macrophages *in vivo*. Preincubation with macrophages *in vitro* did not enhance the response to it (Unanue, 1969). Presumably when such antigens are inoculated in soluble form, they are rapidly bound to tissue macrophages and are thus presented to the immune system in the same form as when they are bound to macrophages *in vitro*. On the other hand, human serum albumin (HSA) was phagocytized poorly by murine macrophages *in vivo*, and preincubation of HSA with macrophages *in vitro* increased the resultant antibody response about one thousand fold when compared to equal microgram quantities of free HSA (Mitchison, 1969). In contrast to free soluble antigen, macrophage-bound antigen has not been shown to induce tolerance (Unanue, 1972).

In addition to enhancement of a humoral or cellular immune response to antigen, macrophages can influence the type of response elicited. In guinea pigs, Oppenheim

and Seeger (1976) have shown that small doses of tetanus toxoid (0.1 μg), when bound to macrophages and given either intradermally or intracardially, induced delayed hypersensitivity type responses to subsequent challenge. In contrast, soluble antigen given in the same dose, schedule and routes resulted in immediate Arthus reactions, indicating high levels of circulating antibody. Further, two sequential injections of macrophage-bound tetanus toxoid resulted only in stronger delayed hypersensitivity. Boosting with tetanus toxoid alone, on the other hand, resulted in high levels of circulating antibody 2 weeks later (Oppenheim and Seeger, 1976). Thus, in some systems the use of macrophage-bound antigen for immunization results not only in an enhanced response but also in a response qualitatively different from that obtained with free soluble antigen.

Finally, many of the immunodeficiencies of newborn animals appear to be associated with the immaturity of the mononuclear phagocytes. Neonatal mice responded very poorly to sheep erythrocytes when compared to adult animals (Argyris, 1968). However, the antibody response could be augmented considerably if the newborn mice were reconstituted with adult peritoneal exudate cells 2 days before immunization. Since mouse spleen cells have been shown to contain antigen sensitive cells 24 hr after birth (Carter and Rector, 1972), the deficiency in neonatal mice would not appear to be in this population of cells. Furthermore, adoptive transfer studies of neonatal and adult mouse spleen cells into X-irradiated recipients also suggested that newborns lacked cells needed to process the antigen, and that these cells were radioresistant, indicating that they might be macrophages (Carter and Rector, 1972). Hardy *et al.* (1973) found that the antibody response in mice to sheep erythrocytes as well as to *Shigella paradysenteria* was not evident until 3–5 days after birth. In exploring the role of the macrophage, they found the PEC from neonatal mice preincubated with *Shigella* antigen were unable to induce an antibody response in adult mice; while the use of PEC from adult mice led to significantly higher titers of antibody in the recipients. From this they concluded that the macrophages from newborn mice were incapable of taking up and processing the *Shigella* antigen sufficiently to induce a detectable antibody response. Blaese (1975) performed studies on the antibody response to several antigens in newborn rats and found that the response of the animal depended on the type of antigen used for immunization. Rats 18 hr old did not respond to sheep or burro erythrocytes, KLH or type III pneumococcal polysaccharide, but responded fairly well to killed *Brucella abortus*. If the sheep erythrocytes given to the neonate were mixed with unfractionated PEC or macrophage-enriched PEC from adults, there was a strong antibody response. Antibody responses to KLH or burro erythrocytes could be induced in neonates in a similar fashion. It is of interest that *Brucella* as well as several other macrophage activators, when injected into newborn rats with the immunogen, were able to substitute for adult macrophages, presumably by activating the resident macrophages. Finally, by inoculating neonates with high doses of antigen it was relatively easy to induce tolerance to that antigen. Blaese (1975) suggested that this was also due to the immaturity of the macrophages in these animals, since the rats did not become tolerant when antigen was injected with adult macrophages.

2.2. ENHANCEMENT OF THE IMMUNE RESPONSE IN VITRO

In vivo experiments such as those described above have shown that macrophages could enhance and, in some cases, qualitatively alter the expression of the immune response. Although demonstrating the significance of macrophages in eliciting various immune responses, such *in vivo* experiments do not allow definition of the mechanisms of action. Unlike experimental models of T cell or B cell deficient animals (e.g. the nude mouse or bursectomized chicken), no model of complete and selective macrophage deficiency is readily available. Macrophages are functionally radioresistant so even in adoptive transfer experiments, resident macrophages may participate in the immune response. Agents which are considered to be selectively

toxic for macrophages such as carrageenan, silica (Allison *et al.*, 1966), or trypan blue (Hibbs, 1974) are less than totally effective (Sawicki and Catanzaro, 1975). In addition, in *in vivo* experiments, it is usually difficult to identify the phase in the immune response during which macrophages are required. For example, in the study of Schwartz and Leskowitz (1969), in which simultaneous injection of carrageenan with antigen temporarily suppressed delayed hypersensitivity responses in guinea pigs, one cannot distinguish between the effect of carrageenan on macrophages involved in the afferent or the efferent limb of the response. In contrast, *in vitro* experiments allow more precise dissection of the site of action of macrophages.

2.2.1. The Macrophage Requirement for the Generation of Antibody-Producing Cells In Vitro

There are several techniques for generating antibody responses in a totally *in vitro* environment. One of the most commonly used techniques is the Mishell-Dutton culture system (Mishell and Dutton, 1966), followed by a Jerne plaque assay (Jerne and Nordin, 1963). Mosier (1967), using a modification of such a system, was one of the first investigators to show a requirement for two distinct cell types for the *in vitro* primary generation of antibody-forming cells (AFC) in response to sheep erythrocytes (SRBC). After separating murine spleen cells on the basis of adherence to a glass Petri dish, neither the nonadherent nor the adherent fraction could generate antibody-forming cells. However, the recombined fractions responded as well as the unfractionated population. These results have been verified with antibody production to all heterologous RBC tested (Katz and Benacerraf, 1972; Pierce, 1969, 1973; Feldmann and Palmer, 1971; Sjobert *et al.*, 1972; Shortman and Palmer, 1971; Shortman *et al.*, 1970; Cosenza *et al.*, 1971; Mosier, 1968; McIntyre and Pierce, 1973). In addition, the macrophage requirement for AFC production has been demonstrated using hapten-protein conjugates (Feldmann *et al.*, 1974) or synthetic soluble copolymers (Pierce *et al.*, 1974; Kapp *et al.*, 1973) as antigens. IgM, IgG and IgA plaque-forming responses have all been shown to require macrophages *in vitro* (Pierce, 1973). Although early reports suggested that the secondary response was not dependent upon macrophages (Pierce, 1969; Katz and Unanue, 1973), it has now been shown that both primary and secondary responses require adherent and phagocytic cells (reviewed by Oppenheim and Rosenstreich, 1976). Whether other cells without known immunologic effects can substitute for macrophages in these *in vitro* responses is not entirely clear. Katz and Unanue (1973) have reported that DNP-KLH bound to fibroblasts could induce an AFC response comparable to that induced with DNP-KLH bound to macrophages when added to nonadherent DNP-KLH-primed mouse spleen cells in a Mishell-Dutton culture system. Similarly, Möller *et al.* (1976) have recently shown that fibroblasts could reconstitute the primary AFC response of nonadherent mouse spleen cells in a Mishell-Dutton culture system with SRBC. In these cases, however, it is likely that residual macrophages left in the spleen preparations after the macrophage-depletion procedures were responsible for inducing the responses observed and that the fibroblasts simply amplified the response by improving the culture conditions by a nonspecific feeder effect. Be that as it may, in addition to adherent cells and B cells, T cells are required for the generation of AFC to the above antigens *in vitro*. In contrast, another class of antigens which can stimulate AFC *in vitro* in the absence of T cells (T-independent antigens) have until recently been considered macrophage independent (Feldmann *et al.*, 1974; Mosier *et al.*, 1974; Feldmann, 1972a; Lemke *et al.*, 1975). These findings suggested that the primary role of macrophages in enhancing AFC production to T-dependent antigens might be related to the requirement for T cells. However, recent reports (Chused *et al.*, 1976; Lee *et al.*, 1976; Pike and Nossal, 1976), showing that some T-independent antigens do in fact require macrophages, do not support this notion. Polyclonal antibody responses to B cell mitogens such as LPS (Lemke *et al.*, 1975) which are also T-independent, have not (with one exception) been shown to require macrophages *in*

vitro (Oppenheim and Rosenstreich, 1976; Rosenstreich and Oppenheim, 1976). The exception is a report by Kragnoff *et al.* (1974) which showed that the LPS-induced proliferative response of B cells from the Peyer's patches of nude mice required either 2-mercaptoethanol or macrophages. However, in this case, T cells were also required, suggesting this situation was unusual if not unique.

2.2.2. *The Requirement for Macrophages for T Cell Responses In Vitro*

Consistent with the enhanced immunogenicity afforded by macrophage-bound antigen in induction of delayed hypersensitivity responses *in vivo* (Oppenheim and Seeger, 1976) are experiments showing that macrophages are required for the generation of T cell responses *in vitro*. T cell proliferative responses in mixed leukocyte culture (MLC) were found to require macrophages in mice (Bevan *et al.*, 1974) and humans (Jones, 1971). Similarly, the *in vitro* generation of cytotoxic effector cells to alloantigens in MLC systems [cell-mediated lympholysis (CML)] has been shown to require macrophages in mice (Wagner *et al.*, 1972). However, whether macrophages function to enhance the immunogenicity of alloantigens is unclear. An alternative explanation for the requirement for macrophages in MLC is that the primary stimulating cell in the MLC is the allogeneic macrophage. This has been observed with MLC in guinea pigs (Greineder and Rosenthal, 1975b), humans (Rode and Gordon, 1974) and rats (Oehler and Herberman, 1977). In these studies, syngeneic macrophages did not reconstitute the MLC responses abrogated by depletion of macrophages from both stimulating and responding populations.

T cell proliferative responses to nonspecific mitogens have also been shown to require macrophages. Rosenstreich and Oppenheim (1976) have recently reviewed evidence that T cell proliferative responses to Concanavalin A (Con A) and phytohemagglutinin (PHA) in guinea pigs are macrophage dependent. Nonadherent human peripheral blood mononuclear leukocytes (PBL), purified by passage over a column containing glass beads, have a diminished proliferative response to PHA which could be reconstituted with as few as 1 per cent phagocytic cells (Oppenheim *et al.*, 1968). Both Oppenheim *et al.* (1968) and Lohrmann *et al.* (1974) have shown that the dependence of human lymphocytes on monocytes for strong proliferative responses to PHA is much easier to demonstrate when suboptimal concentrations of PHA are used. Our laboratory has recently separated human PBL on the basis of adherence by passage of the cells through Sephadex G-10 columns (Jerrells, Dean and Herberman, unpublished observations) and determined the reactivity to PHA and Con A. We found that populations depleted of adherent cells, with less than 1 per cent esterase positive cells, were essentially unresponsive to Con A, whereas the responses to PHA were diminished less consistently, if at all. Reconstitution of the nonadherent cells with irradiated adherent autologous cells which were enriched for esterase positive cells (monocytes) resulted in enhanced PHA and Con A responses, equal to or even greater than the response of the original untreated population. Similarly, in other experiments, we have observed that human PBL forming rosettes with SRBC (E-RFC), when separated from non-rosetting cells by centrifugation in a Ficoll-Hypaque density gradient, may proliferate little if at all in response to Con A (Dean *et al.*, 1977; West, Dean and Herberman, unpublished observations). As observed with the adherent cell-depleted populations described above, the PHA response was diminished less than the Con A response. Approximately 1 per cent of the cells prepared in this way stained positive for esterase or ingested latex particles, and essentially none of the cells had detectable surface IgM. When such populations were further depleted of cells bearing an Fc receptor by adherence to monolayers of antibody-coated SRBC attached to Petri dishes with poly-L-lysine (West *et al.*, 1977), the nonadherent population (consisting of cells lacking the Fc receptor and esterase) also failed to respond to PHA. When reconstituted with various concentrations of adherent autologous PBL, the response to both mitogens was restored. These observations suggest that human PBL require adherent cells, presumably macrophages, to proli-

ferate in response to Con A and that fewer such cells, or a particular subpopulation of adherent cells bearing an Fc receptor, are needed for responses to PHA. Blaese *et al.* (1977), in studies with human PBL, have provided evidence that different macrophage-dependent *in vitro* responses may have varying requirements for macrophages. By using a series of macrophage depletion techniques, they could show a hierarchy of macrophage dependence for several *in vitro* responses. Monocyte-mediated antibody-dependent cell-mediated cytotoxicity (MMADCC) was the assay system most dependent on macrophages. Since macrophages are the effector cells in this assay, even minimal decreases in the percentage of macrophages were associated with decreased cytotoxicity. As more efficient macrophage depletion techniques were used, the synthesis of immunoglobulin induced by pokeweed mitogen (PWM) was depressed. Only the most efficient means of macrophage depletion caused depression of the proliferative response to soluble antigens (*Candida* and SK-SD). Although less dependent on macrophages than MMADCC and the PWM response, the proliferative response to soluble antigens was still shown to be macrophage-dependent. In contrast to our findings, these investigators could not demonstrate a macrophage requirement for a proliferative response to PHA even by using a Shortman column (Shortman, 1968), their most effective technique, to deplete the adherent cells. All of these data indicate that the proliferative response of human PBL to optimal concentrations of PHA requires very few macrophages indeed.

T cell proliferation in response to the immunological adjuvant *Bacillus Calmette-Guérin* (BCG) *in vitro* requires the presence of macrophages (Mokyr and Mitchell, 1975). Mouse thymus cell suspensions, which contain approximately 0.25 per cent macrophages, responded vigorously to BCG, incorporating ³H-thymidine 25–35-fold more than unstimulated controls. When macrophages were depleted to <0.25 per cent (undetectable by Giemsa stain), the response to BCG was abolished. There was no diminution of the response to Con A, indicating viability of the nonadherent thymus cells, and reconstitution of macrophages to 0.5 per cent completely restored the response to BCG. Reduction of macrophages in the spleen from 10 to 0.5 per cent caused no change in two experiments, but significant augmentation of ³H-thymidine incorporation in response to BCG in two others (Mokyr and Mitchell, 1975). Thus, a critical concentration of macrophages 0.25–0.5 per cent was required for the mitogenic effects of BCG on T cells, with higher concentrations tending to cause diminished reactivity of lymphocytes.

T cell proliferative responses induced by sodium periodate or neuraminidase-galactose oxidase (NG) require macrophages in guinea pigs (Greineder and Rosenthal, 1975a). In this system, treatment of either macrophages or T cells alone with these materials resulted in strong proliferative responses when the cells were recombined. In contrast, purified lymphocytes treated with periodate or NG and also pretreated with mitomycin C to prevent their own proliferation could not induce a T cell proliferative response when added to unfractionated guinea pig lymph node cells. These observations further confirm the requirement for interaction between T cells and macrophages in the generation of proliferative responses to nonspecific stimuli.

Proliferative responses of primed T cells to soluble protein antigens have been shown to require macrophages in several systems. In guinea pigs immunized with ovalbumin (OVA) in complete Freund's adjuvant (CFA), lymph node cells depleted of adherent cells did not proliferate in response to the antigens unless reconstituted with adherent cells (Seeger and Oppenheim, 1970; Walderon *et al.*, 1973; Rosenthal and Shevach, 1973; Paul *et al.*, 1976). In addition, purified macrophages, but not lymphocytes preincubated with purified protein derivative of tuberculin (PPD) or OVA and added to purified lymph node cells from immunized animals, induced strong proliferative responses *in vitro* (Rosenthal and Shevach, 1973; Paul *et al.*, 1976). Similarly, Cline and Swett (1968) have shown that when human PBL from tuberculin-sensitive donors were depleted of adherent cells, no blastogenic response was observed in the presence of PPD. Addition of adherent cells with the characteristics of monocytes to the cultures of purified PBL and PPD resulted in strong proliferative

responses. Viable monocytes were required and separation of the responding lymphocytes from the monocytes with cell-impermeable Millipore membranes prevented the enhancing effect. Similar findings using human PBL have been reported by Blaese *et al.* (1972).

Primary T cell proliferative responses to soluble protein antigen *in vitro* have been difficult to demonstrate. However, recently antigen-pulsed macrophages were used to prime purified guinea pig LNC *in vitro* for subsequent proliferative responses upon re-exposure to antigen-pulsed macrophages (Thomas and Shevach, 1976). As will be discussed, these types of experiments have allowed a more detailed understanding of the macrophage-lymphocyte interaction than was previously possible in experiments where T cells were primed *in vivo*.

2.2.2.1. *The requirement for macrophages for the production of lymphokines in vitro.* In addition to the induction of T cell division, antigens and mitogens induce the production by T cells of numerous mediators termed lymphokines. *In vitro* systems have been developed to investigate the events leading to lymphokine production. Thus far, all such *in vitro* systems show that macrophages are required for the production of mediators by T cells.

Migration inhibition factor (MIF) production by guinea pig lymphocytes primed with CFA required macrophages pulsed with PPD (Ohishi and Onoue, 1975). In this case, the macrophage requirement could be replaced by supernatants of macrophages stimulated with lipopolysaccharide (LPS) (Ohishi and Onoue, 1976). Similarly, our laboratory (Landolfo *et al.*, 1978) has recently shown that spleen cells from C57BL/6 mice bearing Moloney sarcoma virus-induced tumors could be induced to produce MIF by culture with 3 M KCl extracts of tumor cells bearing relevant antigens. Spleen cells depleted of macrophages by adherence techniques did not produce MIF when cultured with the 3 M KCl extract. However, when purified macrophages from light mineral oil-induced peritoneal exudates of normal syngeneic mice were added to the adherent cell-depleted spleen cells in the presence of free antigen, MIF production could again be demonstrated. Further, spleen cells with macrophages which were preincubated with soluble tumor antigens for 2-6 hr and washed to remove all unbound antigen could also produce MIF. More recently, we have shown that only antigen-pulsed spleen cells containing macrophages compatible with the responding cells at the major histocompatibility complex (MHC) could produce MIF (Landolfo *et al.*, 1977). Unlike the findings of Ohishi and Onoue described above for the PPD system in guinea pigs, the macrophage requirement in the MSV tumor system has been shown to require cell-cell contact and could not be replaced by supernatants of LPS-stimulated macrophages (Landolfo, Holden and Herberman, unpublished observations).

In addition to MIF, a number of other mediators are produced by stimulated T cells *in vitro*. Macrophages were required for interferon production by PBL from tuberculin-sensitive human donors in response to PPD *in vitro* (Epstein *et al.*, 1971a). Unprimed human lymphocytes stimulated with PHA produced interferon only in the presence of macrophages (Epstein *et al.*, 1971b). Osteoclast-activating factor (OAF) produced by human PBL in response to PHA required adherent cells (Horton *et al.*, 1974). In guinea pigs, T cell production of macrophage chemotactic factor by primed T cells stimulated with soluble antigen required macrophages (Wahl *et al.*, 1975). In sensitized humans, the production of fibroblast chemotactic factor *in vitro* by PBL stimulated with the sensitizing antigen required the presence of adherent cells (Postlethwaite *et al.*, 1976).

In summary, all immune responses involving triggering of T cells, whether to help B cells respond to T-dependent antigens, to help other T cells to become effector cells, or to mediate effector functions themselves, require at least small numbers of macrophages. In our discussion we have made no effort thus far to distinguish between those macrophage requirements dependent on cellular contact with T cells and the ability of macrophage products to effectively replace the actual adherent cell.

Similarly, for most of the systems discussed, we have made no distinction between those T cell responses shown to require macrophages which share some region in the MHC and those in which no genetic restriction has been described. Oppenheim and Rosenstreich (1976) have recently reviewed these issues and have presented evidence suggesting that T cell responses to nonspecific mitogens and cellular antigens, i.e., to alloantigens in MLC or CML or heterologous erythrocyte antigens such as SRBC, do not require the presence of intact and viable macrophages in the culture, but instead only require soluble factors from macrophages such as lymphocyte-activating factor (LAF) (Gery *et al.*, 1972). These factors act, with the cellular antigens or mitogens, as 'second signals' analogous to second signals required for B cell activation to promote T cell triggering and to amplify the response (Rosenstreich and Oppenheim, 1976). Several lines of evidence account for these views. Antibody production to heterologous erythrocytes *in vitro* required T cells and macrophages as well as B cells as previously described. However, Calkins and Golub (1972) have shown that macrophages could provide the helper effect even when separated from macrophage-depleted spleen cells by a cell-impermeable Millipore filter. The antigen (SRBC in this case) and not the macrophages had to be in contact with the responding cells. Further, Chen and Hirsch (1972; and reviewed by Chen and Hirsch, 1975) have shown that the requirement for macrophages in a Mishell-Dutton culture system could be replaced entirely with various thiols, of which the most widely used is 2-mercaptoethanol (2-ME). Addition of such agents to lymphoid cells in tissue culture has long been known to enhance the viability of the cells remaining after several days in culture (Fanger *et al.*, 1970; Click *et al.*, 1972). When even small numbers of macrophages were added to nonadherent mouse spleen cells in the absence of 2-ME, the viability was shown to be similarly increased (Pierce and Kapp, 1976). However, Pierce and Kapp (1976) have suggested that rather than replacing macrophages in Mishell-Dutton cultures, 2-ME simply augments the activity of the small number of macrophages left in culture after macrophage depletion procedures. This, of course, may be difficult to directly demonstrate until cell populations can be depleted of macrophages so efficiently that they will not respond to cellular antigens under optimal conditions even in the presence of 2-ME.

T cell responses to cellular alloantigens in MLC and CML have also been shown to be supported by factors from cultures of macrophages (Bach *et al.*, 1970) and by 2-ME in mice (Bevan *et al.*, 1974). As discussed earlier, in guinea pigs, rats and humans, macrophages must be present only in the stimulating cell population as the primary stimulating cells. In this respect, they play a dual role of both presenting the alloantigens to the responding T cells as well as nonspecifically supporting the response (Rosenstreich and Oppenheim, 1976). In addition to T cell responses to alloantigens, T cell proliferative responses to nonspecific mitogens can also be supported by macrophage factors. In the presence of macrophage products, even purified T cells respond well to PHA (Rosenstreich and Oppenheim, 1976). Similarly, the Con A response of T cells requires only macrophage factors. In the situation where macrophage products or thiols such as 2-ME can replace the requirement for macrophages, there is obviously no necessity that the macrophages producing the factors share MHC components with the responding T cells.

In contrast to the T cell responses to cellular antigens and mitogens just discussed, T cell responses to soluble protein antigens have been shown to require the actual presence of viable macrophages in the culture system (for review, see Rosenstreich and Oppenheim, 1976). For these cellular interactions, the MHC does appear to play an important role. This will be discussed in detail in the section on antigen presentation (Section 2.3.2).

2.3. MECHANISMS OF ACTION

The immunogenicity of many soluble protein antigens appears to be correlated with the ability of *in situ* macrophages to take up and process the antigen (discussed above

in Section 2.1). Antigens not taken up well *in vivo*, and therefore poorly immunogenic, could be preincubated with macrophages and injected as macrophage-bound antigen. Antigens treated in this way were highly immunogenic and never produced tolerance. B cell tolerance to T-dependent antigens (all soluble protein antigens are T-dependent) has been associated with the prolonged exposure of the B cells to free antigen in the absence of activated helper T cells (for review, see Klaus *et al.*, 1976). One component of the mechanism of increased immunogenicity associated with macrophage-bound antigen may be that antigens administered in this way are less likely to be exposed to B cells in free form than are antigens injected without macrophages. All T cell responses to soluble protein antigens, either production of mediators or proliferative responses, require viable macrophages. In addition to preventing prolonged exposure of B cells to antigens, the evidence suggests that macrophages are also needed to present soluble protein antigens to T cells. With these points in mind, we can first discuss antigen processing by macrophages and then the ability of macrophages to present antigens to T cells.

2.3.1. Antigen Processing

Most antigen picked up by macrophages *in vitro* is endocytosed and broken down into nonimmunogenic fragments (Unanue, 1972; Ellner and Rosenthal, 1975). A small percentage of the antigen associated with the macrophage remains on the cell surface and is highly immunogenic (Unanue, 1972). In mice, the antigen on the surface of macrophages is trypsin-sensitive and can be detected by specific antibody (Unanue *et al.*, 1969b). Further, when mouse macrophages were preincubated with soluble protein antigens and then exposed to antigen-specific antibody or were trypsinized prior to *in vivo* inoculation, the resulting antibody response was largely abrogated (Unanue, 1972). In guinea pigs the observations are more complex. Calderon *et al.* (1974) have provided evidence that guinea pig macrophages pulsed with PPD at 4° and then trypsinized while at 4° were no longer immunogenic. In contrast, when guinea pig macrophages were pulsed with PPD at 37°, only a fraction of the immunogenicity was trypsin sensitive. Further, when macrophages were pulsed at 4° and then incubated at 37° before trypsinization, the macrophage-associated immunogenicity became progressively more trypsin resistant. This led these investigators to postulate two steps in antigen processing by macrophages. The first consisted of antigen binding to the macrophage surface, which was trypsin-sensitive and temperature-independent. The second step involved either internalization or sequestration of the immunogenic component in the folds of the membrane rendering it relatively insensitive to trypsinization. Similarly, Ellner and Rosenthal (1975) have shown that antigen taken up by guinea pig macrophages was resistant to trypsin and could not be detected by specific antibody. They have postulated that the macrophage-lymphocyte interaction might induce a return of the antigen to the surface of the macrophage where it would be available for presentation to T cells. Calderon and Unanue (1974) studied the handling of KLH by macrophages and found that a small percentage (3–7 per cent) of the antigen picked up by macrophages *in vitro* was internalized and slowly released into the medium as a smaller molecule than the original antigen, and that part of this fragment was still reactive with anti-KLH antisera. This protein release was associated with live macrophages and was trypsin-resistant. In addition, its release was independent of protein synthesis.

In summary, there is general agreement that the majority of the antigen phagocytized by macrophages is degraded into nonimmunogenic fragments and released. This may play an important role in preventing tolerance induction. In addition, a small percentage of the antigen is maintained on the surface of the macrophages where it is highly immunogenic. In mice, the antigen is trypsin-sensitive and detectable by specific antibody. In guinea pigs, the immunogenic fragments are trypsin-resistant and cannot be detected by specific antibody, suggesting they may be sequestered in the membrane. Finally, some of the immunogenic portions of the molecule may be

internalized, to be slowly released in an immunogenic form for a variable length of time. The biochemical events involved in these processes are largely unknown.

2.3.2. Antigen Presentation

2.3.2.1. *Antigen presentation by macrophages: effects on T cells.* Whereas antigen processing concerns the effects of macrophages on antigens which result in altered and frequently enhanced immunogenicity as compared to the native form, antigen presentation is concerned with the macrophage-lymphocyte interaction which results in antigen-specific triggering of lymphocyte responses. The most recent concepts of antigen presentation by macrophages grew largely out of studies of the genetic control of the immune response. Certain strains of mice (Benacerraf and McDevitt, 1972), guinea pigs (Paul and Benacerraf, 1974) and rats (Kunz and Gill, 1974) have been found to be high or low antibody responders to specific antigens. The ability to mount a strong antibody response to many antigens was linked to the MHC (Benacerraf and McDevitt, 1972; Kunz and Gill, 1974). By use of recombinant strains of mice for genetic mapping, the immune response (Ir) genes have largely been found to be located in the I region of the MHC although in some cases the H-2K region has not yet been ruled out (Shreffler and David, 1975). Ir gene expression, or the ability to generate immune responses to antigens under Ir gene control, was once thought to be primarily a property of T cells (Paul and Benacerraf, 1974). Now, however, macrophages in the guinea pig (Shevach, 1976) and the rat (Tada *et al.*, 1976) and probably B cells, in some cases in mice (Mozes and Shearer, 1971), are thought to express Ir genes. The I region of the MHC also codes for I region-associated (Ia) cell surface antigens found on the membranes of subpopulations of T cells, B cells and macrophages as well as some nonlymphoid cells (Hammerling, 1976). That Ia antigens are important in macrophage-T cell interactions is indicated by the fact that antisera directed toward these Ia antigens have been shown to block T cell proliferative responses to macrophage-bound soluble antigen (Shevach and Rosenthal, 1973; Shevach, 1976). Further, recent evidence has been presented that antisera directed toward Ia antigens coded for in the I-J subregion of the MHC (recently described by Murphy *et al.*, 1976), when preincubated with macrophages but not the macrophage-depleted responding lymphocytes, could block the *in vitro* humoral response to burro red blood cells (BRBC) (Neiderhuber and Shreffler, 1976). Studies of lymphoid cellular interactions have suggested that T cell-B cell collaboration *in vitro* and *in vivo* required identity at various I subregions of the MHC (Katz *et al.*, 1973a, 1976). Similar requirements have been suggested for the ability of macrophages to present antigens to T cells for helper cell induction (Erb and Feldmann, 1975b, 1976) and for induction of antigen-specific T cell proliferative responses (Rosenthal and Shevach, 1973). However, exceptions to this rule have been reported. Pierce *et al.* (1976) and Kapp *et al.* (1973) have shown that completely allogeneic macrophages can prime T cells *in vitro* to become helper cells for antibody-forming cell (AFC) production to GAT (a random terpolymer of L-glutamic acid-L-alanine-L-tryosine). In addition, Heber-Katz and Wilson (1976) have shown rat T cells, 'filtered' through irradiated F₁ hybrid rats to deplete alloreactive cells, could collaborate with allogeneic B cells in a primary *in vitro* anti-SRBC response. These experiments, then, suggest that Ia compatibility may not be required for cellular interactions of unprimed cells.

Similar experiments have been performed *in vivo*. Bechtol *et al.* (1974) have used tetraparental (allophenic) chimeric mice constructed from the fusion of mouse embryos of strains which are high (CWB) and low (C3H) responders to Poly L-(Tyr, Glu)-poly-D, L-Ala-poly-L-Lys (TGAL). These strains are congenic on the C3H background, differing for the Ig allotype and the H-2 complex. The percentage of each parental lymphoid cell type in the adult chimeric mice could be determined by allotype analysis of the serum immunoglobulin. Those mice with a high percentage of the CWB Ig allotype proved to be high responders to TGAL and those with a low percentage of the CWB allotype were poor responders. This suggested that the

genetic responsiveness was still associated with the predominant lymphoid cell type in the chimeric mice. However, when high responder chimeric mice were immunized with TGAL, the anti-TGAL response consisted of antibody of both allotypes and the ratio of the CWB allotype to the C3H allotype had not changed from the resting state. This suggested that B cells of both haplotypes responded to CWB T cell help, thus providing evidence that T-B collaboration could occur *in vivo* despite MHC differences. These observations were further supported by the experiments of von Boehmer *et al.* (1975a). They adoptively transferred, into lethally irradiated F₁ mice, SRBC primed T cells from tetraparental bone marrow chimera mice which were depleted of cells from the other parental donor by treatment with alloantisera and complement. The recipients were then inoculated with SRBC and with SRBC-primed B cells depleted of T cells by anti-theta plus complement from the opposite parental strain. These allogeneic, primed T and B cells cooperated well to give a strong anti-SRBC response. Since these investigators (von Boehmer *et al.*, 1975b) had previously demonstrated the lack of alloreactivity between T cells from chimeric mice and cells from mice of one of the parental strains, the observed response was not due to an allogeneic effect. Similarly, Bechtol *et al.* (1976) have provided convincing evidence that the anti-TGAL responses (discussed above) in C₃H ↔ CWB tetraparental mice were in no way due to an allogeneic effect. These experiments have clearly shown that, at least in some situations, T cells and B cells can collaborate *in vivo* despite I region differences. To provide an explanation for these observations, Pierce *et al.* (1976) have recently pointed out that the Ia identity requirement had been seen in experiments using primed cells in secondary responses, whereas those investigators not observing the Ia identity genetic restriction had tested either primary responses or responses of T cells primed in a chimeric environment (Kapp *et al.*, 1973; Pierce *et al.*, 1976; Heber-Katz and Wilson, 1975). Further, Pierce *et al.* (1976) demonstrated that T cells primed *in vivo* with allogeneic macrophages required macrophages syngeneic to those used for priming, to trigger a secondary response. Moreover, macrophages syngeneic to T cells primed with allogeneic macrophages could not be substituted for allogeneic macrophages for the secondary challenge. These findings are supported by the work of Miller *et al.* (1976a), using an adoptive transfer of delayed hypersensitivity (DTH) system in mice. They have shown that T cells of one parental haplotype, sensitized in a chimeric environment, could adoptively transfer DTH to either of the parents despite total histoincompatibility. Further, T cells from (BALB/c × CBA)F₁ hybrid mice, sensitized in either BALB/c or CBA nude mice, could transfer DTH reactivity only to the parent syngeneic to the nude mouse in which they were sensitized (Miller *et al.*, 1976a). They previously showed that F₁ cells sensitized in an F₁ environment could transfer DTH reactivity equally well to either parent (Miller *et al.*, 1976b). These experiments support the notion that the original macrophage-T cell interaction is responsible for genetic restrictions observed for macrophage interactions with primed T cells. More direct evidence for this hypothesis has been provided by recent reports of Thomas and Shevach (1976, 1977a, b). They showed that purified lymph node cells of (strain 2 × strain 13)F₁ guinea pigs, primed *in vitro* with OVA-pulsed macrophages from either parent, could be restimulated only with macrophages of the same parent pulsed with the same antigen. In contrast, F₁ LNC primed with F₁ macrophages would respond to either of the parental macrophages in a secondary response (Thomas and Shevach, 1976). These experiments, however, only showed priming of F₁ LNC by macrophages, albeit with interesting genetic restrictions for subsequent T cell re-stimulation. To examine priming of T cells with totally allogeneic macrophages, they had to pretreat the macrophages with specific anti-Ia antisera to block alloantigen sensitization which would otherwise result in a strong secondary MLC upon re-exposure of the allogeneic macrophages to the allosensitized T cells. They found that the antisera, in addition to blocking the Ia determinant on the macrophages, served as an antigen to which the T cells could be sensitized. Once primed, the T cells responded secondarily only to macrophages of the same haplotype which were treated

with anti-Ia antisera. Neither macrophages of the proper haplotype without pretreatment with anti-Ia, nor syngeneic macrophages regardless of treatment with anti-Ia stimulated. Therefore, this represents a clear example of T cell priming with histoincompatible macrophages, the secondary response of which was specific not only for the antigen but also for the haplotype of the macrophage used for the initial priming. Further, these investigators found that antisera toward the B.1 MHC determinant (analogous to the H-2 K and D regions in the mouse) also could act as an antigen and that the Ia genetic restriction for secondary stimulation was operative in this case also. On the basis of these data, they have suggested that protein antigens bound to Ia and other MHC molecules on macrophages serve as 'complex antigenic determinants' to which T cells are primed. Moreover, they concluded that Ia antigens must indirectly influence the presentation of antigen bound to other MHC molecules since only anti-B.1 treated macrophages with Ia determinants identical to those of the anti-B.1 treated macrophage used for priming could elicit a secondary proliferative response (Thomas and Shevach, 1977a). In addition, they have shown that TNP-modified macrophages could specifically sensitize allogeneic T cells, but only after the alloreactive clone of T cells was depleted by treatment with BUdR and light during a primary MLC. The reason for this was not obvious, but since not even syngeneic macrophages, modified with TNP, could sensitize T cells during a MLC proliferative response, it was likely that a suppressor mechanism was being generated which was dependent on the proliferation of the alloreactive cells (Thomas and Shevach, 1977b).

Paul *et al.* (1976) have recently shown that guinea pig LNC primed *in vivo* with OVA could be restimulated *in vitro* with antigen-pulsed macrophages from either parent, and if during the proliferative phase of the secondary response induced by such antigen-pulsed macrophages, the responding cells were treated with BUdR and exposed to light, thus eliminating the proliferating clone, the remaining cells could be stimulated only with OVA-pulsed macrophages from the alternate parent. Positive selection experiments showed that if F₁ LNC, primed *in vivo*, were restimulated with OVA-pulsed macrophages of one parent, the responding cells were then hyper-responsive for OVA-pulsed macrophages of that parent but only modestly responsive to OVA-pulsed macrophages of the other parent.

All of these data can be explained by the hypothesis that the original macrophage-T cell MHC relationship established a preference in the secondary response for macrophage MHC antigen which induced the primary response. I, D and K region identity primary sensitization, T cells appear to recognize antigens in complex or association with MHC antigens on macrophages. For secondary stimulation then, the primed T cell requires presentation of antigen in complex or association with the same macrophage MHC antigen which induced the primary response. I, D, and K region identity requirements for secondary T cell stimulation have been demonstrated depending on the antigen used (Miller *et al.*, 1976a; Paul *et al.*, 1976; Thomas and Shevach, 1976; Pierce *et al.*, 1976). In the case of F₁ cells sensitized in an F₁ environment, or parental cells sensitized in a chimeric environment, two clones of antigen-reactive cells may be generated, one for each macrophage haplotype expressed during the sensitization.

In summary, these recent observations indicate that MHC gene products on macrophages play a critical role in the sensitization process of the T cell and that T cell receptors recognize antigen in complex or association with macrophage Ia or other MHC determinants. Paul *et al.* (1976) have postulated that the MHC has evolved as the basis for cellular interactions which are essential for the competence of the immune response. This interesting hypothesis serves to point out the rather central role of macrophages in induction of T cell-dependent immune responses.

2.3.2.2. Antigen presentation by macrophages: effects on B cells. In contrast to the recent advances in understanding of the macrophage-T cell interaction, the macrophage-B cell interaction has received little attention. Rather, the T cell-B cell interaction has been stressed (Katz *et al.*, 1973a, b, 1976; reviewed in Katz and Benacerraf, 1972). The demonstration that T-independent B cell antigens were also

macrophage independent (Shortman *et al.*, 1970; Mosier *et al.*, 1974; Feldmann, 1972a, b) has suggested that the primary or sole role of macrophages is to sensitize T cells and only through that mechanism could macrophages influence B cell responses. However, recent demonstrations that B cell responses to some T-independent antigens either require or are greatly enhanced by macrophages (Chused *et al.*, 1976; Lee *et al.*, 1976; Pike and Nossal, 1976) suggest that macrophages may play a role in B cell activation and that their effect on B cells in these responses may have been overlooked. There is, in fact, some evidence that activated macrophages produce factors that can stimulate B cells directly to respond to T-dependent antigens in the absence of T cells (Schrader, 1973; Wood and Gaul, 1974; Hoffman *et al.*, 1976a, b; Wood and Cameron, 1975). In addition, there is evidence that T cell helper factors may require macrophages to activate (or provide a second signal for) B cells. Feldmann (1972c) has reported experiments done in double chambered Marbrook vessels in which primed T cells, separated from T cell-depleted spleen cells by a 0.1 μ Nucleopore membrane, could induce specific AFC production by the T cell-depleted spleen cells in the presence of the priming antigen (DNP-KLH). When macrophages were also depleted from the T cell-depleted spleen cells, no specific AFC production could be demonstrated. Reconstitution of the spleen cells with purified anti-theta and complement-treated PEC (macrophages) again resulted in specific AFC production. Further, when primed T cells were cultured in the upper chamber of the Marbrook vessel with DNP-KLH, and purified PEC were cultured alone in the lower chamber, Feldmann (1972c) found that the thoroughly washed PEC could induce a strong antigen-specific AFC response when combined with T cell-depleted spleen cells in a secondary culture devoid of additional antigen. From such experiments, Feldmann (1972c) concluded that macrophages were required to present the antigen-T cell factor complex to B cells. Schrader and Feldmann (1973) arrived at similar conclusions during investigations of antigenic competition. In these experiments, mice which had been primed with fowl gamma globulin (FGG) were injected with donkey RBC (DRBC) i.p. or i.v. 2 days before serving as donors of spleen cells to be used in a Marbrook-Diener culture system to produce AFC to DNP coupled to FGG. They found that the AFC response was very poor when DNP-FGG was used as an antigen, but when DNP was coupled to a thymus-independent antigen such as flagella (FLA), a strong AFC response was observed. This indicated that the B cells were fully competent to produce antibody when triggered by a T-independent antigen and suggested the defect in the response to DNP-FGG was in either the T cells or the macrophages. They found that addition of very small numbers of normal macrophages to the system (0.2 per cent of the responding cells) allowed a normal AFC response, compared to the response of cells from FGG-primed mice not preinjected with DRBC. In addition, the ability to respond to DNP-FGG was restored by trypsinizing the responding cells prior to culture. From these experiments, they concluded that macrophages in the spleens of mice pre-injected with DRBC were unable to present the T cell helper factor to the B cells due to the pre-injection with DRBC *in vivo*. The trypsinization, they concluded, acted to remove a surface inhibitor from the surface of the macrophages.

Further evidence that macrophages alone can enhance B cell antibody production was provided by Opitz *et al.* (1976). They showed that normal mouse spleen cells would generate a polyclonal antibody response in a Mishell-Dutton culture system if cocultivated with high concentrations of mouse PEC for 8 hr before depleting the spleen cells of all phagocytic cells and reculturing them in media containing 2-ME. Control cultures not cultured with high numbers of mouse PEC generated no such response.

In summary then, these observations suggest that macrophages can have significant effects on B cell responses to both T-dependent and T-independent antigens. In view of the recent work describing the mechanisms of antigen presentation to T cells by macrophages (previous section), it seems likely that macrophages may also be involved in the presentation of T cell-dependent helper factors to B cells. Alter-

natively, primed T cells, upon activation by antigen, may liberate factors which activate macrophages which then provide a second signal to B cells for specific AFC production. Considering the changing concepts of the genetic restrictions (or lack thereof) on T cell-B cell collaboration and macrophage-T cell interactions, it would seem that further investigations are needed on the role of macrophages in the relationship between T cells and B cells as well as their role in stimulating B cells directly.

3. SUPPRESSION OF IMMUNE RESPONSES BY MACROPHAGES

In the previous sections we have discussed evidence that macrophages can enhance immune responses *in vivo* and *in vitro*. Various mechanisms were discussed, both in terms of the effect of macrophages on the antigen itself and the requirement for macrophages of various lymphocyte responses to antigens and mitogens. In most cases, normal macrophages in very low concentrations were used to demonstrate these enhancing effects.

In this section, we shall concentrate on suppressive effects of macrophages on the immune response. In contrast to the enhancing effects, the suppressive effects have usually been seen with greater concentrations of macrophages or with highly activated macrophages. First, we shall describe a series of basic observations made in our laboratory and in others which provide a framework on which a more general concept of immunosuppression resulting from the inhibition of lymphocyte proliferation by cells of the reticuloendothelial system (RES) can be constructed. What is known about the mechanism of macrophage-mediated cytostatic effects will be reviewed. We suggest that 'activation' of macrophages is a relative term and that the degree of suppression is related not only to the activation state of the macrophages but also to the number of macrophages in the proximity of an expanding clone of lymphocytes. We shall discuss various ways in which macrophages can become activated and thereby become more suppressive. An important recent finding which will be emphasized is that T cells can generate suppressor macrophages via a factor isolated from Con A-induced suppressor T cells. Therefore, suppressor T cells may exert their effects indirectly through activation of suppressor macrophages.

We shall then discuss the evidence that suppressor macrophages *in vitro* and an activated reticuloendothelial system (RES) *in vivo* can be shown to have similar effects on lymphocyte responsiveness. A number of examples of suppressed immune responses associated with an activated RES will be discussed, along with evidence that in many cases the cell which mediates the suppressor effect is probably an activated macrophage. Finally, we shall discuss the observations that macrophages, usually considered to be nonspecific in their effects, may in fact be able to distinguish between neoplastic and non-neoplastic cells.

3.1. BASIC OBSERVATIONS

Our laboratory has recently investigated a number of cases of suppressed immune responses *in vitro* and, in many instances, has identified the responsible agent as a macrophage (Kirchner *et al.*, 1974a, b, c, 1975a, b; Oehler *et al.*, 1977a, b; Fernbach *et al.*, 1976a, b; Glaser *et al.*, 1976; reviewed in Kirchner *et al.*, 1976). Many of the original observations were made by Kirchner *et al.* (1974a, b, 1975a). It was initially observed that spleen cells from C57BL/6 mice bearing tumors induced by inoculation with Moloney sarcoma virus (MSV) responded poorly if at all to T cell mitogens such as PHA and Con A and frequently had a depressed response to the B cell mitogen LPS (Kirchner *et al.*, 1974a, b). There were several possible explanations for such findings. If T cells, and occasionally B cells, migrated from the spleen during the growth of the tumor, the lack of cells even potentially capable of responding to mitogens when cultured *in vitro* could account for the defect. However, purification of the lymphocytes by passing the spleen cells from MSV tumor-bearing mice (MSV

spleen cells) over a rayon adherence column [a technique by which cells capable of ingesting latex particles were largely depleted but the ratio of surface immunoglobulin (Ig) positive to Ig negative cells was essentially unchanged (Kirchner *et al.*, 1974a)] reconstituted the proliferative response to the mitogens (Kirchner *et al.*, 1974a, b). Therefore, cells capable of responding to the mitogens were present in the spleen. Thus two possible explanations remained: either the potentially responsive cells were diluted by an influx of cells which would not respond to mitogens (Smith and Konda, 1973), or a population of adherent cells was present which somehow prevented the potentially responsive cells from proliferating. To test whether a suppressive effect was playing a role in the failure of the MSV spleens to respond to mitogens, spleen cells from MSV tumor-bearing mice were mixed with spleen cells from normal mice and the mixture was incubated with PHA. Virtually no proliferative response was observed in the cell mixture, demonstrating the role of suppressor cells (Kirchner *et al.*, 1974b). To characterize the cell or cells responsible for the suppressive effect, a number of subpopulations of cells from MSV spleens were tested for their suppressive effects on the proliferative response of normal spleen cells to PHA. Pretreatment of the MSV spleen cells with anti-theta and complement to remove T cells had no effect or actually increased the suppressive effects (Kirchner *et al.*, 1974b, 1975a). In contrast, depletion of adherent cells by passage over a rayon column, or of phagocytic cells by pretreatment with iron powder and magnet, totally abrogated the suppressive effect (Kirchner *et al.*, 1974b). In addition, pretreatment of the cells with carrageenan, which is specifically toxic for macrophages and not for lymphocytes (Lake *et al.*, 1971), largely abrogated the suppression (Kirchner *et al.*, 1975a). Further, the suppressor activity was resistant to 2500 r X-irradiation (Kirchner *et al.*, 1975a). These data indicated that the MSV spleen cells contained a subpopulation of radioresistant, phagocytic, carrageenan-sensitive, non-T cells, and thus probably macrophages or monocytes, which could suppress the proliferative response of lymphocytes to mitogens (reviewed in Kirchner *et al.*, 1976). Subsequent reports concerning studies of tumor-bearing rats (Glaser *et al.*, 1975; Veit and Feldman, 1976b), humans (Broder *et al.*, 1975) and mice (Pope *et al.*, 1976) confirmed that suppressed responses to mitogens could be due to the presence of a population of suppressor macrophages. In addition to the suppression of mitogen responses, macrophage-mediated suppressive effects have been demonstrated in a variety of other *in vitro* reactions. Fernbach *et al.* (1976b) found that MSV spleen cells responded poorly to alloantigens in MLC and CML. When mixed with normal mouse spleen cells, irradiated MSV spleen cells were strongly suppressive compared to the effect of adding equal numbers of irradiated normal spleen cells. When MSV spleen cells were pretreated with carrageenan or when the phagocytes were depleted with iron and magnet, the suppressive effect was no longer seen. In addition, the cells were found to be adherent and radioresistant. As expected, treatment with anti-theta and complement had no effect. Addition of peritoneal exudate cells (PEC), induced with thioglycolate and purified by Petri dish adherence so that greater than 95 per cent of the cells were macrophages morphologically, also resulted in suppression of the MLC and the CML (Fernbach *et al.*, 1976a). Berlinger *et al.* (1976) have shown that depressed MLC responses of PBL from some cancer patients could be restored by passing the PBL over a Sephadex G-10 column which in their hands selectively depleted monocytes. Kirchner *et al.* (1975a) found that the T cell proliferative response of spleen cells from tumor-bearing mice to tumor-associated antigens in a mixed lymphocyte-tumor interaction (MLTI) was greatly improved by depletion of adherent or phagocytic cells. Similarly, Glaser *et al.* (1975) showed that spleen cells taken from rats bearing progressively growing tumors were largely unresponsive to tumor-associated antigens in a MLTI. After depletion of adherent or phagocytic cells, positive responses were detected. We recently confirmed and extended these observations, and have shown that the suppressor cells in tumor-bearing rats were resistant to treatment with a specific heterologous anti-rat thymocyte serum and complement

(ATS and C) as well as to a heterologous anti-rat immunoglobulin serum and complement (Oehler *et al.*, 1977a). These data indicated that the suppressor cells in spleens of tumor-bearing rats which inhibit specific lymphoproliferative responses to tumor cells *in vitro* were macrophages. In rats, however, we found that normal spleen cells added to MLTI cultures as third parties were also suppressive, albeit not as suppressive as spleen cells taken from tumor-bearing donors. Extensive characterization procedures showed suppressor cells in the spleens of non-tumor-bearing rats similarly were macrophages. These experiments indicated that the suppressive nature of macrophages may not necessarily represent a pathologic state but rather reflect a normal regulatory mechanism which is intensified in some pathological conditions.

Injection of mice with agents such as heat-killed *Corynebacterium parvum* (*C. parvum*) results in a strong stimulation of the reticuloendothelial system (RES) (Halpern *et al.*, 1963; Halpern, 1975). Splenic macrophages from mice treated with *C. parvum*, strongly suppress the lymphoproliferative responses to PHA, Con A and LPS, and, if given in a high enough dose, to pokeweed mitogen (PWM) (Scott, 1972; Kirchner *et al.*, 1975c; reviewed in Kirchner *et al.*, 1976). Mitchell *et al.* (1973) have shown similar effects after treatment of mice with Bacillus Calmette-Guerin (BCG).

In contrast to the inhibitory effects of suppressor macrophages on proliferative responses and on the generation of cytotoxic lymphocytes which require proliferation (Cantor and Jandinski, 1974; Peavy and Pierce, 1975), cytotoxicity by previously-generated effector cells seems not to be affected. Primary T cell-mediated cytotoxicity to tumor-associated antigens (Kirchner *et al.*, 1974b), PHA-induced T cell-mediated cytotoxicity (Kirchner *et al.*, 1975a) and antibody-dependent cell-mediated cytotoxicity (Poplack *et al.*, 1976; reviewed in Kirchner *et al.*, 1976) appear to be unaffected by suppressor macrophages. Thus, immune cell functions which are independent of proliferation may not be sensitive to suppression by macrophages. It seems likely that a major mechanism of action of suppressor macrophages is inhibition of lymphocyte proliferation, with consequent limitation of the expansion of an antigen-sensitive clone of effector lymphocytes. Keller (1974a) has emphasized the need for macrophages to be activated to mediate the suppressor effect. His data show that on a per cell basis, peptone broth-induced rat peritoneal macrophages are more suppressive than non-induced peritoneal macrophages. However, in sufficient concentrations, 'resting' or non-induced macrophages also inhibited lymphocyte proliferation (Keller, 1974a). Our laboratory has provided further evidence, in both mice (Fernbach *et al.*, 1976a) and rats (Oehler *et al.*, 1977b), that by simply increasing the concentration of PEC in MLC-CML cultures, lymphoproliferative responses and the resultant generation of cytotoxic effector cells were inhibited without affecting the viability of the cells in the suppressed cultures. Further, the distinction between 'activated' and 'resting' macrophages is complicated by the fact that many substances including lymphocyte mediators (reviewed by David, 1975), with which circulating macrophages must to a variable degree be in contact *in vivo*, activate macrophages. Therefore, it may be better to think of relative degrees of activation rather than of an all or none phenomenon. This would account for both Keller's (1974a) findings that activated macrophages were more suppressive than resting (less activated) macrophages and our observations that increased numbers of peritoneal macrophages added to CML cultures as third parties [5-8 per cent in rats (Oehler *et al.*, 1977b) and greater than 10 per cent in mice (Fernbach *et al.*, 1976a)] were strongly suppressive. In contrast, addition of a thirty fold greater number of X-irradiated thymocytes to rat CML cultures as third parties had no suppressive effect, suggesting that cell crowding was not the cause of suppression by higher numbers of macrophages (Oehler *et al.*, 1977b). Thus, it would appear that macrophages, either low numbers of strongly activated cells or higher numbers of minimally activated cells, can suppress lymphocyte proliferation and in that way inhibit or abrogate the expansion of a clone of sensitized lymphocytes.

3.2. MECHANISMS OF ACTION

3.2.1. Assay Systems

Much of the information concerning the mechanisms of macrophage-mediated suppression has come from the work of Keller (1974a, b, 1976; Keller *et al.*, 1975) who measured the rate of proliferation of antigen- or mitogen-triggered lymphocytes in Petri dishes containing various concentrations of purified macrophages obtained from rat peritoneal exudates. Basically, Keller demonstrated that high macrophage to lymphocyte ratios (10:1) completely inhibited proliferation of the lymphocytes (Keller, 1975). The effect was nonspecific in that the proliferation of syngeneic, allogeneic, and xenogeneic lymphocytes could be suppressed. Moreover, use of malignant lymphoma cells as targets of suppression has provided a consistent and simpler assay system for analysis of the mechanism of the macrophage-mediated suppression (Keller, 1974a), since these cells did not need to be stimulated to proliferate.

Support for the use of rapidly dividing tumor cells as indicator cells for suppressor macrophage activity has recently been provided by our laboratory (Oehler *et al.*, 1977a). We have directly compared the ability of macrophages to suppress the proliferation rate of lymphoma lines maintained *in vitro* to their ability to suppress specific lymphoproliferative responses of immune spleen T cells to tumor-associated antigens in a mixed lymphocyte-tumor interaction (MLTI). Under the proper conditions, results obtained in one assay could be used to predict results obtained in the other. This indicated that the cytostatic mechanism was similarly effective on rapidly proliferating lymphoid cells regardless of the stimulus (Oehler *et al.*, 1977a).

3.2.2. Metabolic Requirements for Macrophage-Induced Suppression

Keller (1974b) has evaluated the effects of various agents and treatments to determine the metabolic requirements for macrophage-induced suppression. The suppressive effect was sensitive only to protein synthesis inhibitors and inhibitors of glycolysis (Keller, 1974b). When various parameters of macrophage activity were monitored, such as cellular adherence, pinocytosis, bactericidal capacity, glucose oxidation, production of RNase and DNase, and others, only alterations in the rate of protein synthesis correlated with the cytostatic effects observed (Keller, 1975). Pretreatment of macrophage monolayers with inhibitors of DNA synthesis such as mitomycin C and X-irradiation had no effect on their cytostatic activity. In addition, the suppressor activity was resistant to treatment with a variety of enzymes including trypsin, neuraminidase and pronase. Inhibitors of pinocytosis or phagocytosis had no effect. Moreover, careful microscopic examination of the experimental cultures showed no relationship of increased phagocytic activity to the degree of suppression. Thus, although the mechanisms by which macrophages inhibit lymphoproliferative responses are unknown, the available evidence suggests that the ability to suppress can be correlated with the rate of protein synthesis and not with various other parameters of macrophage activation. This could be interpreted to suggest that only a subpopulation of macrophages, characterized by its increased rate of protein synthesis, is suppressive or that only macrophages which have been activated to undergo increased protein synthesis are suppressive. However, until these findings are confirmed in other laboratories, it may be premature to speculate on the mechanisms by which suppressor macrophages mediate their effects.

3.2.3. Suppressor Effects of Macrophages Mediated by Soluble Factors

Although there are a number of reports describing suppressor effects of macrophages which require cell-cell contact (Sjöberg and Britton, 1972; Sjöberg, 1971; Eggers and Wunderlich, 1975; Kirchner *et al.*, 1975a; reviewed in Kirchner *et al.*, 1976), macrophages also appear to mediate suppressor effects through the effects of soluble factors (for review see *Modulation of Immunity by Soluble Mediators* by Byron H. Waksman, in this series). Suppressor factors produced by or dependent on

macrophages have been described by a number of investigators. Calderon *et al.* (1974) have described a factor recovered from cultures of mouse peritoneal exudate cells which inhibited ^3H -TdR incorporation by, and proliferation of, tumor cells and mitogen-stimulated lymphocytes *in vitro*. It was of low molecular weight and resistant to trypsin digestion. Further, it could be absorbed by tumor cells cultured *in vitro*. Similarly, Waldman and Gottlieb (1973) have described a low molecular weight factor isolated from rat PEC which inhibited proliferation of antigen-stimulated and resting rat lymphocytes. Krakauer *et al.* (1976) have demonstrated a soluble suppressor factor which inhibited immunoglobulin production of PWM-stimulated mouse spleen cells. This factor was apparently produced by macrophages in the ascitic fluid of hypogammaglobulinemic mice bearing plasmacytomas. Similarly, Kolb *et al.* (1977) have demonstrated a suppressor factor apparently originating from macrophages of mice bearing large plasmacytomas which inhibited the PFC response to SRBC in Mishell-Dutton cultures.

Caution must be exercised, however, in evaluating the effects of soluble factors produced from lymphoid cells in tissue culture. When only one parameter such as ^3H -TdR incorporation is used as a measure of suppression, the possibility that breakdown products of dying cells are present in the culture which compete with radiolabeled DNA precursors and thus inhibit ^3H -TdR incorporation without inhibiting cell proliferation must be ruled out. One group (Opitz *et al.*, 1975a), in fact, has performed a series of biochemical characterizations on a soluble factor which they had previously shown to suppress ^3H -TdR incorporation of PHA-stimulated mouse lymphocytes (Opitz *et al.*, 1975b) and conclusively demonstrated that it was thymidine. There are several ways to avoid such tissue culture artifacts. One way is to use a second assay system independent of ^3H -TdR incorporation to demonstrate the suppressive effect. Fernbach *et al.* (1976a) showed that macrophages suppressed the generation of cytotoxic effector cells and the appearance of blast cells as well as the incorporation of ^3H -TdR by mouse T cells in MLC. In this study, by observing the effects of various concentrations of macrophages on the MLC, they noted that ^3H TdR incorporation was inhibited at a slightly lower concentration of macrophages than was the generation of lymphoblasts or the generation of cytotoxic effector cells. It seems likely that the slightly disparate findings were a result of the presence of cell breakdown products in the media. This effect, however, was minor compared to the effect of greater numbers of macrophages which totally suppressed all three parameters of the response generated in the MLC.

In addition to using alternate assay systems, one can measure the incorporation of other labeled protein or nucleic acid precursors such as tritiated uridine, valine or leucine. Opitz *et al.* (1975a) showed that the incorporation of these precursors was not inhibited by supernatants from cultures containing cell breakdown products which artifactually inhibited ^3H -TdR incorporation. Similarly, Fernbach *et al.* (1976a) have suggested that the use of low specific activity ^3H -TdR (300 mCi/mM instead of 6 Ci/mM) minimized the effect of artifactual ^3H -TdR incorporation inhibition. Perhaps the easiest way to avoid such artifacts is to wash the cells immediately before addition of ^3H -TdR, thus removing any cell breakdown products which may have accumulated during the culture period.

Thus, in light of the findings of Opitz *et al.* (1975a), who identified their low molecular weight suppressor factor as thymidine, it seems mandatory that any soluble suppressor substance which inhibits ^3H -TdR incorporation of proliferating cells *in vitro* must be shown to actually inhibit cellular proliferation to be seriously considered as a factor which may have immunosuppressive activity *in vivo*.

3.2.4. Macrophage Activation as a Result of Interaction with Exogenous Agents or with Lymphocytes and Their Mediators

As previously discussed, macrophages which are in states of increased activation, usually associated with increased rates of protein synthesis, are more suppressive on a per cell basis than less activated macrophages. A number of agents are known to

stimulate the reticuloendothelial system (RES) which results in the appearance of highly activated macrophages in the organs of the lymphoid system and particularly in the spleen. Similar effects on the activation of macrophages by such agents have been observed *in vitro*. First, we will discuss exogenous agents which activate macrophages and the little that is known about the mechanisms involved. Then we will discuss possible mechanisms by which suppressor macrophage activity may be enhanced or regulated by interaction with lymphocytes or their mediators during the induction of an immune response.

Several agents capable of activating macrophages are known. Bacterial products can activate macrophages. Shilo (1959) has reviewed evidence that in the case of gram negative bacteria, the lipopolysaccharide component of the cell wall, and particularly the lipid A component, is a potent macrophage activator. Other macrophage activators include certain organisms such as *C. parvum* (Halpern *et al.*, 1964), *Listeria monocytogenes*, BCG, *Mycobacterium tuberculosis* (reviewed by McGregor and Logie, 1975) and double-stranded RNA (reviewed by Allison and Davies, 1975). The actual mechanism of macrophage activation by these agents is unknown. *C. parvum* has been extensively studied to learn why it is such a powerful RES stimulant. Prévot (1975) has suggested that a component of the bacterial cell wall is responsible for the intense RES activation and has tentatively named it 'reticulostimulin'. Efforts to purify reticulostimulin, however, have largely resulted in abrogation of the macrophage-activating effect (Adlam *et al.*, 1975) and thus have been unsuccessful. In addition to microbial agents such as *C. parvum*, most soluble adjuvants are known to stimulate reticuloendothelial proliferation and to activate macrophages (Unanue *et al.*, 1969a). Spitznagel and Allison (1970) have correlated the ability of various substances to labilize lysosomes in macrophages with their adjuvant effects. They found that retinol (vitamin A alcohol) and LPS were particularly active in this respect. Despite the large number of empirical observations that the administration of various agents such as those mentioned above results in RES activation, very little is known about the actual mechanisms involved. It seems likely that a greater understanding of the mechanisms of this important aspect of the immune response will enable such agents to be used as one of the means of intervening in and regulating the immune response in the future.

In addition to interaction with exogenous agents, macrophage-lymphocyte interactions also have been shown to result in activation of macrophages. Similar to the requirement for macrophages for the sensitization phase of most immune responses (discussed earlier in this review), macrophages have also been shown to be required to mediate the effector phase of the response in collaboration with sensitized lymphocytes in some cases. McGregor and Koster (1971) have shown that lasting immunity to *Listeria monocytogenes* in rats can be adoptively transferred with thoracic duct lymphocytes, but cannot be mediated without an intact macrophage system. Conversely, purified macrophages from immune animals could not transfer lasting immunity even to intact animals. These results suggest that certain immune effector functions require an interaction between immune T cells and macrophages which neither cell alone can carry out regardless of the immune status. Nathan *et al.* (1973) have studied mediators from T lymphocytes which affect macrophages. They found macrophage migration inhibition factor (MIF) to be particularly active in increasing macrophage adherence, phagocytosis, and glucose carbon 1 oxidative hexose monophosphate shunt pathway metabolism. Moreover, they found that interaction of macrophages with MIF also results in increased protein synthesis. Two to three days of exposure to the lymphokine *in vitro* was required before the effects became detectable in their system (Nathan *et al.*, 1971). In a recent review of the effect of lymphocyte mediators on macrophages, David (1975) has confirmed and extended the reports of Nathan *et al.* (1973). He discussed evidence that, in addition to the metabolic and morphologic effects on macrophages attributed to MIF by Nathan *et al.* (1973), MIF also decreased lysosomal enzymes, increased the cytoplasmic enzyme lactic dehydrogenase, as well as membrane adenylate cyclase, and

increased glucosamine incorporation. More importantly, bacteriostasis to *Listeria monocytogenes* and tumoricidal activity have been shown to be strongly enhanced.

Thus, there is ample evidence that macrophages can be activated by interaction with a number of agents including lymphocyte mediators, the best studied of which is MIF. As a result of interaction with MIF, various parameters of macrophage activation such as the rate of protein synthesis were found to be increased. As we have discussed, the rate of macrophage protein synthesis correlated quite well with the suppressor activity of macrophages. Therefore, it is obvious that a number of agents, including products of activated lymphocytes (at least MIF), are capable of augmenting suppressor macrophage activity.

3.3. *The Relationship of Suppressor T Cells to Suppressor Macrophages*

Recent reports of suppressor cells other than macrophages include the specific suppressor T cells of Tada and Taniguchi (1976) and the allotype suppressor T cells described by Herzenberg *et al.* (1976). In addition, Dutton (1972) has shown that Con A-stimulated T cells can nonspecifically block anti-SRBC responses in Mishell-Dutton culture systems *in vitro*. Similarly, Peavy and Pierce (1974) have used Con A-stimulated T cells to nonspecifically suppress the generation of cytotoxic effector cells to alloantigens in MLC-CML reactions. The mechanism of action in both cases is thought to be a suppression of the proliferative event required for the expression of the effector function (Dutton, 1972; Peavy and Pierce, 1974). However, these studies have not demonstrated whether suppressor T cells act directly on the responding lymphocytes or via another cell type such as a macrophage. As discussed above, activated macrophages (suppressor macrophages) have been shown to suppress *in vitro* lymphoproliferative reactions. Gershon (1974), in an exhaustive review of suppressor T cells, has allowed that the role of macrophages in the actual inhibition of proliferation resulting from suppressor T cell activity is uncertain. Recently, a suppressor factor has been isolated from Con A-induced suppressor T cell culture supernatants. This factor suppressed the generation of anti-SRBC responses in *in vitro* Mishell-Dutton cultures. The factor has been termed 'SIRS' for soluble immune response suppressor (Rich and Pierce, 1974). Analysis of the factor has revealed that it is a glycoprotein secreted by the Con A-stimulated T cell with a molecular weight of 48,000-67,000 daltons (Tadakuma *et al.* (1976a). SIRS appeared to be distinct from immunoglobulins in that it did not bind to immunoabsorbant columns coated with anti-mouse IgG or IgM (Tadakuma *et al.*, 1976a). More recently Tadakuma *et al.* (1976b) have demonstrated that SIRS acts not directly on B cells or T cells, but requires macrophages to mediate its effect. Thus far, SIRS has been indistinguishable from MIF (Tadakuma *et al.*, 1976b). Nathan *et al.* (1973) have reported that MIF is a potent macrophage activator, causing enhanced protein synthesis. As discussed above, Keller (1975b) has shown that macrophage suppressor activity correlated best with the rate of protein synthesis as a measure of activation. Thus, it seems likely that SIRS may generate suppressor macrophages and thereby exert its immunosuppressive effect *in vitro*. There may not be two separate types of suppressor cells, i.e. suppressor T cells and suppressor macrophages, but rather the actual suppressor cell may always be a macrophage with T cell dependence for some forms of activation. Folch and Waksman (1974) have shown that normal rat spleens contain suppressor cells which can suppress the proliferative response in mixed leukocyte cultures. The effector cells mediating the suppression were adherent, but cells from donors having undergone adult thymectomy, irradiation and bone marrow reconstitution were inactive. They concluded that the suppressor cell was an adherent suppressor T cell. We have recently confirmed that normal rat spleens contain adherent cells which suppress MLC and CML reactivity (Oehler *et al.*, 1977b). However, we have also shown that the suppressor cells are phagocytic and *resistant* to treatment with a specific anti-T cell serum and complement. Our data indicate that the final effector cell mediating the suppression is a macrophage. To reconcile our findings with those of Folch and

Waksman (1974), we have suggested that the activated macrophages in normal rat spleens are a reflection of, and dependent on, T cell activity *in vivo* (Oehler *et al.*, 1977c). The early findings of McGregor and Koster (1971) showing a macrophage requirement for the adoptive transfer by immune thoracic duct lymphocytes of immunity to *Listeria monocytogenes* may be explained in a similar manner. In this case, the immune T cells may have led to the gradual development of activated macrophages with anti-microbial activity.

3.4. EVIDENCE THAT SUPPRESSOR MACROPHAGES CAN SUPPRESS IMMUNE RESPONSES IN VIVO

3.4.1. *Introductory Remarks*

Most of the clear-cut evidence that macrophages can act as suppressor cells comes from *in vitro* observations. It is rather difficult to directly relate such *in vitro* findings to the role of these cells in regulating the immune response *in vivo*. Part of this reflects the general problem of determining the *in vivo* role of *in vitro* phenomena. A classical approach in this direction has been to examine the effects of adoptive transfer of cells with a particular function *in vitro*. However, adoptive transfer of suppressor macrophages seems unlikely to confer a suppressed state on the recipient for at least two reasons. Firstly, highly activated macrophages undoubtedly do not stay highly activated long after transfer. Nathan *et al.* (1973) showed that macrophages activated by lymphocyte mediators *in vitro* remained activated only 24–48 hr after removal of the activating agent. Secondly, adoptively transferred, strongly activated macrophages might lodge at the site of inoculation or some other unwanted location and not migrate into the same lymphoid microenvironment as the lymphocytes engaged in the immune response or even into the appropriate lymphoid organs. Local adoptive transfer of macrophages, either admixed with responding lymphocytes or inoculated into the site of the immune response (e.g. draining lymph node), would be expected to overcome these problems. However, little evidence along these lines has been reported. The main approach which has been made to evaluate the *in vivo* role of macrophages is to administer agents which can stimulate the reticulo-endothelial system (RES) and cause the activation and migration of macrophages into the lymphoid and circulatory systems. In addition, various pathological conditions, both experimentally induced and naturally occurring which are associated with RES stimulation, present opportunities to observe the effect of strongly activated macrophages in high cell densities in the proximity of lymphocytes during the generation of an immune response *in vivo*. There are a number of studies showing a depressed immune response in the presence of either a high macrophage cell density or a highly activated RES. Based in part on the *in vitro* evidence discussed above, one might predict that immune responses requiring the generation of an expanding clone of antigen-sensitized lymphocytes would be suppressed if sufficient suppressor macrophage activity were present during the proliferative phase of the response. Interpretation of such studies, however, is complicated by the fact that the stimulating agents have *in vivo* effects other than activation of macrophages, and also that macrophages are involved at many levels of the immune response, both in initiating immune responses, i.e. antigen presentation, antigen processing, prevention of tolerance, and frequently in the final effector phases of the immune response, i.e. delayed hypersensitivity (Scott, 1974), tumor (Remington *et al.*, 1975; Hibbs *et al.*, 1972; Evans and Alexander, 1972) and tissue graft rejection (Evans and Grant, 1972), and protection from certain infectious agents (Gadebusch, 1972). It has been possible to design experiments to take advantage of the fact that macrophage participation in various phases of an immune response to an antigenic challenge do not necessarily occur simultaneously. Shortly after antigenic challenge, macrophages aid in sensitizing lymphocytes. Then, after a lag period which varies from 24–28 hr to several days, depending on the antigen, the lymphoproliferative phase begins, followed by the appearance of the effector phase of the immune

response, i.e. antibody production, cytotoxic T cell appearance, memory cell distribution and/or macrophage activation. Thus, the lymphoproliferative phase occurs some time after antigen inoculation and continues through the time of the appearance of the effector aspect of the response. To demonstrate suppressive effects of macrophage activation, the optimal time of most intense macrophage activation should be during the proliferative phase, i.e. some time after antigen inoculation and before the appearance of effector cells (plasma cells or cytotoxic T cells). Similar to the kinetics of the immune response, the length of time between injection of a stimulating agent and the appearance of maximal macrophage activation must be taken into account. The kinetics of the appearance of suppressor macrophages in the spleen of experimental animals differs slightly with the particular activating agent used, the dose and the route of administration.

Thus the most convincing evidence that suppressor macrophages can inhibit the generation of an immune response *in vivo* would come from experiments in which suppressor macrophages and few proliferating lymphocytes have been shown to be in lymphoid organs at the time when one would expect the lymphoproliferative phase of the immune response to be occurring. Ideally, one might then find that upon removal or inactivation of suppressor macrophages, fully immunocompetent lymphocytes remain. In addition, *in vivo* administration of agents which inactivate macrophages without interfering with their numerous enhancing effects would be expected to reverse the immunosuppression. Such evidence would point to suppressor macrophages as the main or only cause for the suppressed immune response observed *in vivo*.

In the sections to follow, we shall review the available evidence for *in vivo* suppression of immune responses by macrophages. In addition, evidence will be reviewed regarding the possibility that some forms of antigenic competition may be due to the appearance of splenic suppressor macrophages.

3.4.2. *Suppression Directly Related to Macrophage Concentration In Vivo*

A simple but still somewhat artificial way to observe the effects of local adoptive transfer of high macrophage concentrations on antigen-triggered lymphocytes *in vivo* would be to place antigen-primed lymphocytes in a small Millipore chamber with the antigen and a large number of macrophages, and implant the chamber into the peritoneal cavity of a lethally irradiated histocompatible mouse. Measurement of the resultant serum and chamber antibody levels and comparison with levels generated in mice in which similar chambers were implanted without high numbers of macrophages would give some indication of the effect of the extra macrophages.

Using such an experimental design, Perkins and Makinodan (1965) placed SRBC-primed mouse spleen cells in 0.1 μm Millipore chambers with SRBC and various numbers of peritoneal exudate cells and implanted them in irradiated mice. As controls, they implanted chambers to which they added either unprimed mouse spleen cells in numbers equal to the primed responding cells plus SRBC or primed responding cells plus SRBC alone. After 9 days, they measured the hemolysin titer in both the chamber fluid and the serum. The antibody titers from mice bearing chambers containing primed spleen cells plus SRBC were very similar to those with primed spleen cells, SRBC and equal numbers of unprimed spleen cells. In contrast, the chamber fluid or sera from mice bearing chambers containing additional PEC sufficient to make up 20–30 per cent of the primed responding cell had markedly depressed titers. No concentration of PEC produced enhanced antibody production and high numbers of PEC completely suppressed the response. To reduce the artificiality of the approach with Millipore chambers, similar experiments were designed in which mice were injected intraperitoneally with glycogen to increase the number and the level of activation of the peritoneal macrophages, or only with saline. Both groups were challenged with SRBC *i.p.* and the serum hemolysin titers measured every other day for 10 days. At all time points tested, mice pretreated with glycogen

had lower serum antibody levels compared to the saline-injected controls. The objective of these experiments was to obtain *in vivo* data to support the notion that macrophages enhanced the SRBC response, but the results which were in the opposite direction indicated that high cell densities of macrophages in the environment of lymphocytes which were stimulated to proliferate and produce antibody could suppress antibody production *in vivo*. Hoffman (1970), using an *in vitro* Mishell-Dutton culture system, obtained similar results and, in addition, provided evidence that the suppressive effect of the high concentration of macrophages was mediated through an inhibition of the proliferative phase of the response. He found that the optimal macrophage concentration for an *in vitro* SRBC response was 2 per cent of the responding spleen cells. Adding purified peritoneal macrophages to a final concentration of 20 per cent of the responding spleen cell population resulted in profound inhibition of the response. Moreover, analysis of the cells in the suppressed cultures revealed greatly decreased blastogenesis and plasma cell formation. He postulated that the high concentration of macrophages inhibited the proliferation of antigen-sensitive cells, thereby preventing a clonal expansion of cells destined to produce antibody. Alternatively, since T cells are also required to provide a helper factor for the production of antibody to heterologous erythrocytes, both *in vitro* and *in vivo* (reviewed in Oppenheim and Rosenstreich, 1976; Katz and Benacerraf, 1972), the suppressive effect of macrophages could have been mediated through inhibition of the T cell response. However, there is little if any evidence that macrophages can suppress the production and release of factors *per se*. So this alternative seems unlikely. Moreover, evidence has been presented that suppressor macrophages can inhibit LPS-induced B cell proliferative responses in mice (Kirchner *et al.*, 1974b) and rats (Yoshinaga *et al.*, 1972). Since LPS induces B cell proliferation directly without the aid of accessory cells (Lipsky and Rosenthal, 1976; reviewed by Oppenheim and Rosenstreich, 1976), it would appear that macrophages are capable of suppressing B cell proliferation directly. Thus, it is likely that the suppression by high concentrations of macrophages of antibody production in the above cases was due to inhibition of proliferation of the antigen-sensitized clone of B cells.

3.4.3. *Suppression of Antibody Production In Vivo by a Macrophage-Activating Agent*

Lipopolysaccharide has been shown to activate macrophages (see Section 3.2.4) and *in vivo* inoculation of this material has been shown to inhibit immune responsiveness (Bradley and Watson, 1964; Franzl and McMaster, 1968). As predicted above, the demonstration of LPS-induced suppression is very dependent on the time of administration of LPS in relation to antigen inoculation. It seems necessary for the LPS to come in contact with macrophages which are closely associated with lymphoid cells prior to exposure to antigen. In other words, if LPS and the antigen (SRBC) were given by the same route, the LPS had to be given 24–48 hr prior to the antigen. Only relatively large doses of LPS induced suppression when given i.p. 24 hr before antigen inoculation into the same area. If LPS was given i.v. 24 hr before antigen i.p., five-fold less endotoxin could totally abrogate the anti-SRBC response (Franzl and McMaster, 1968). Similarly, Bradley and Watson (1964) have shown that a relatively large dose of LPS given to mice at the time of injection of actinophage MSP8 simply delayed the production of neutralizing antibody, whereas daily injections of LPS suppressed the antibody production indefinitely despite continued presence of the phage in the tissues of the animal. Further, hyperimmunized mice with high levels of circulating antibody could be suppressed with daily LPS administration. When the LPS was stopped, the antibody production resumed as indicated by the prompt appearance of high serum levels of neutralizing antibody. The results of these studies indicate that antibody production in mice can be suppressed by an agent which activates macrophages. However, it is possible that the inhibitory effects of LPS are independent of macrophages, since as noted above, LPS can have direct effects on B cells.

3.4.4. *The Suppressive Effect of C. parvum on the Generation of Resistance to Tumor Cells*

C. parvum is a powerful RES-activating agent (Halpern *et al.*, 1963) which can induce splenic suppressor macrophages in mice (Kirchner *et al.*, 1975c; Scott, 1972). Similarly, *C. parvum* injection has been shown to suppress some immune responses requiring proliferation *in vivo*. Immunization procedures which normally conferred a significant amount of protection from tumor challenge were much less effective if instituted 4–5 days after *C. parvum* injection (Smith and Scott, 1972; Woodruff *et al.*, 1976). The studies of Scott (1972) demonstrated the temporal relationship between *C. parvum* injection and the appearance of suppressor macrophages. Intravenous inoculation of *C. parvum* produced maximal suppression of responsiveness of spleen cells to PHA at day 7, with some persisting inhibition through day 28. This suppression was shown to be mediated by macrophages. Considering this time course for the appearance of suppressor cells in the spleen after *C. parvum* injection, one might predict that immune responses generated in the spleen and dependent upon the rapid expansion of an antigen-sensitized clone of lymphocytes would be suppressed if the proliferative response occurred about 7 days after *C. parvum* injection. Thereafter (for at least 2 weeks) moderate suppression might be observed. Smith and Scott (1972) investigated the protective effects of *C. parvum* injection on challenge of CBA mice with R-1 leukemia cells (a radiation-induced syngeneic leukemia). Inoculation of unimmunized mice with as few as ten tumor cells *i.p.* resulted in the death of all recipients within 10–15 days. When mice were immunized with a single dose of 1×10^5 heavily irradiated R-1 cells 7 days prior to the inoculation of 100 R-1 cells *i.p.*, 50 per cent of the mice survived. When *C. parvum* was given 7 days before the immunizing dose of irradiated R-1 cells, 90 per cent of the mice died within 20 days of the injection of non-irradiated R-1 cells. Similarly, Woodruff *et al.* (1976) have reported that in CBA mice, protection against a syngeneic chemically induced fibrosarcoma by immunization with irradiated tumor cells was decreased by pretreatment with *C. parvum* 5 days before the immunizing dose. Recent studies in our laboratory by Kirchner *et al.* (1975b) have provided insight into possible mechanisms operative in the experiments described above. C56BL/6 mice injected with Moloney sarcoma virus developed a tumor at the site of inoculation which reached maximum size 14 days after inoculation and regressed completely by 21 days (MSV mouse tumor system). Spleen cells taken from donors after tumor regression could be stimulated to become cytotoxic *in vitro* by cocultivation with mitomycin C-treated RBL-5 tumor cells, shown to contain relevant antigens (Herberman *et al.*, 1974). To test the effect of *C. parvum* inoculation in this system, mice were given *C. parvum* *i.p.* 23 days after virus infection, and 7 days later primed spleen cells from these mice were cultured *in vitro* with RBL-5 to generate cytotoxic effector cells. The cytotoxicity generated with such cells was much lower than that generated with spleen cells from immune mice not treated with *C. parvum*. This effect of *C. parvum* appeared to be mediated by suppressor macrophages, since mixture of immune spleen cells with spleen cells from mice inoculated 8–10 days earlier with *C. parvum* resulted in a marked suppression of generation of cytotoxicity. The suppression by the spleen cells from *C. parvum*-treated mice was dependent on the presence of macrophages but not T cells. Bernstein *et al.* (1977) have recently shown that the generation of cytotoxic effector cells from non-cytotoxic primed rat spleen cells required the proliferation of a clone of lymphocytes during cocultivation with mitomycin-treated (C58NT)D tumor cells. Similarly, our laboratory has provided evidence that a proliferative response is also required for the generation of cytotoxic effector cells *in vitro* from non-cytotoxic primed cells in the MSV mouse tumor system (Stiller, Holden and Herberman, unpublished observations). Thus it seems likely that the suppressed cytotoxic response generated *in vitro* by *C. parvum*-pretreated MSV immune mice was due to the inhibition of the required lymphoproliferative response by the *C. parvum*-induced suppressor macrophages. These observations by no means prove that the interference

with tumor resistance (Smith and Scott, 1972; Woodruff *et al.*, 1976) was caused by the inhibition of a proliferative response required to generate an immune state, but the similarities between the *in vivo* observations and the *in vitro* findings are striking. An important issue is whether the increased numbers of activated macrophages found in the spleens of animals treated with *C. parvum* (or other RES stimulants) can inhibit lymphoproliferative responses sufficiently to suppress *in vivo* generation of cytotoxic effector cells. Evidence that spleens of *C. parvum*-treated mice can inhibit the proliferation of antigen-stimulated lymphoid cells *in vivo* has been provided by Howard *et al.* (1967). They treated F₁ hybrid mice with i.v. *C. parvum* 4 days before i.v. injection of 10⁸ viable parental lymphoid cells, thus initiating a graft versus host (GVH) reaction in the environment of a highly stimulated reticuloendothelial system. The pretreatment with *C. parvum* protected the majority of the mice from the uniformly fatal GVH reaction which occurred in the untreated control mice. Evidence for the mechanism of action of the protective effect was obtained by examining the spleens of representative mice from each group for proliferating lymphocytes. Twelve days after GVH initiation, the percentage of donor cells in mitosis was 85–90 per cent in the untreated F₁ hybrid recipients. In contrast, only 0–5 per cent of the proliferating cells were of donor origin in the mice pretreated with *C. parvum*. These data show that *C. parvum* induced an environment in the spleens of mice which suppressed rapidly-proliferating alloantigen-stimulated parental lymphoid cells *in vivo*. It remains to be directly demonstrated that macrophages were in fact responsible for the anti-proliferative effect.

C. parvum and other potent RES stimulators are frequently used as immunotherapeutic agents to increase resistance against tumor growth. However, the observations discussed above indicate that, under some circumstances, these agents may actually interfere with effective anti-tumor immunity and that these immunosuppressive effects may be mediated by suppressor macrophages.

3.4.5. *The Possible Role of Suppressor Macrophages in Antigenic Competition*

The interference with the immune response to an antigen by prior inoculation with high doses of a non-cross-reactive second antigen has been termed antigenic competition. Antigenic competition has been extensively studied and the subject has been thoroughly reviewed recently (Pross and Eidinger, 1974). As suggested by Pross and Eidinger (1974), it seems likely that numerous mechanisms of action are responsible for the various phenomena grouped under the heading of antigenic competition. Since some instances of antigenic competition can actually be shown to be suppressive phenomena and the suppression is at least associated with an inhibition of proliferative response, suppressor macrophages should be considered as one type of mediator of these effects.

Several lines of evidence suggest that some forms of antigenic competition may actually be mediated through a suppressor mechanism rather than direct competition between antigens for responding cells. Sjöberg and Britton (1972) have shown that spleen cells from mice heavily preimmunized with SRBC suppressed the PFU response of normal mouse spleen cells to horse red blood cells (HRBC) when cocultivated in Mishell–Dutton culture systems in the presence of SRBC. Similarly, Thomas *et al.* (1975) have shown that when spleen cells from mice immunized with ovalbumin (OVA) were added with OVA to Mishell–Dutton cultures with SRBC-primed spleen cells the SRBC-specific PFU response was suppressed compared to cultures in which either the OVA or the OVA immune spleen cells were omitted. Further, Thomas *et al.* (1975) reported that in suppressed cultures, ³H-TdR incorporation was depressed compared to nonsuppressed control cultures, indicating that the mechanism of the suppressor effect involved the proliferation rate of the responding cells. Möller (1971a) has presented evidence that DNA synthesis in the spleen of mice subjected to antigenic competition is reduced compared to normal controls. When mice were preimmunized by two injections of HRBC and then irradiated with

900 r before receiving normal spleen cells in adoptive transfer, the rate of DNA synthesis in the spleen as determined by ^{125}I UdR incorporation *in vivo* was suppressed by 40–50 per cent when compared to unimmunized but otherwise similarly treated control mice. Whether T cells or B cells were preferentially suppressed in these systems was not tested. However, there is evidence that the suppressive effect can be observed on either population. Although it is generally accepted that the induction of antigenic competition requires injection of a T-dependent antigen (reviewed in Pross and Eidinger, 1974), the antibody response to T-independent antigens was shown to be susceptible to suppression (Sjöberg and Britton, 1972), suggesting that B cells could be suppressed directly. On the other hand, Bash *et al.* (1976) have shown that spleen cells from rats injected 24 hr earlier with large doses of OVA *i.v.* responded poorly to PHA compared to normal controls, and when mixed with normal spleen cells, these cells were highly suppressive. The experiments showed that the suppressive effect on proliferation *in vitro* could also affect T cells. The apparent lack of specificity of the suppressor effect is consistent with the nonspecific nature of suppressor macrophage activity (reviewed by Kirchner *et al.*, 1976). Bash *et al.* (1976) have shown that the suppressor cell activated by injection of large doses of OVA is adherent and somewhat sensitive to carrageenan [a specific macrophage toxin (Lake *et al.*, 1971)]. However, since low doses of cyclophosphamide given *i.p.* at the time of antigen administration *in vivo* also abrogated the suppressor effect, the authors concluded that T cells and macrophages acted synergistically to be suppressive. Thomas *et al.* (1975) have shown that the suppression of AFC production *in vitro* by OVA immune mouse spleen cells cultured in the presence of OVA was mediated by a soluble suppressor factor produced by T cells. The characteristics of the factor are not unlike those of 'SIRS', another soluble suppressor described by Rich and Pierce (1974) and characterized by Tadakuma *et al.* (1976). SIRS, as discussed above (Section 3.3), is a product of Con A-stimulated T cells which can nonspecifically suppress AFC production to heterologous erythrocytes *in vitro*. The mechanism of action has to some extent been elucidated in that the target cell has been shown to be a macrophage in the responding cell population (Tadakuma *et al.*, 1976). Thus, the role of suppressor macrophages in the experiments reported by Thomas *et al.* (1975) would seem to require further investigation.

In summary, although the mechanisms for antigenic competition are largely unknown and may be quite heterogeneous, in the instances in which suppression of proliferation is associated with the depressed response, it seems likely that suppressor macrophages activated by antigen-stimulated T cells or their products play a role.

3.4.6. *The Role of Macrophages in Immunosuppression Associated with Graft Versus Host Disease*

In mice, antibody responses to SRBC have been shown to be greatly suppressed *in vivo* after induction of chronic GVH disease (Blanden, 1969; Möller, 1971b). Blanden (1969) also has shown that in mice undergoing mild chronic GVH, immunization with KLH in complete Freund's adjuvant was virtually ineffective, as indicated by a greatly depressed immediate hypersensitivity reaction upon challenge with antigen. Similarly, PFÜ responses generated *in vivo* to T-independent antigens have also been shown to be suppressed in mice undergoing chronic GVH. In contrast to the decreased humoral response to the various types of antigens (cellular, soluble, T-dependent and T-independent), the resistance of such mice to infection with *Listeria monocytogenes* was significantly increased (Blanden, 1969). Such increased resistance has frequently been associated with elevated levels of macrophage activity (McGregor and Koster, 1971). As discussed earlier, states of increased macrophage activation *in vivo* have occasionally been associated with immunosuppressive effects. One might therefore predict that normal syngeneic lymphoid cells adoptively transferred into irradiated animals undergoing chronic GVH disease would be immunosuppressed. Möller (1971b) has shown that this is indeed the case. He found that

both primed and normal spleen cells when adoptively transferred into irradiated F_1 mice undergoing chronic GVH disease had strongly suppressed antibody responses to i.v. SRBC when compared to irradiated and adoptively transferred but otherwise normal F_1 control recipients. Byfield *et al.* (1973) showed that viable parental T cells were required in the cells injected to induce GVH disease-related suppression of the response to SIII. However, certain aspects of their findings led them to postulate that macrophages, perhaps activated in response to the alloantigen-stimulated parental T cells, in fact directly mediated the suppressor effect. This was supported by the findings of Sjöberg (1971, 1972). He has shown that spleens taken from F_1 hybrid donors 1 week after induction of GVH disease (GVH spleen cells) were essentially unresponsive to SRBC in Mishell-Dutton cultures. Moreover, when GVH spleen cells were added to normal or primed F_1 or parental spleen cells with SRBC, the response was strongly suppressed compared to F_1 or parental spleen cells cultured with SRBC alone (Sjöberg, 1971). The media from suppressed cultures could support strong anti-SRBC responses of normal spleen cells, indicating the suppression was not due to media exhaustion or a stable soluble factor. Pretreatment of the GVH spleen cells with anti-theta and complement before mixing with normal cells did not abrogate the suppressor activity. Depletion of phagocytes from the GVH spleen cells with iron and magnet treatment did abrogate the suppressor effect, indicating that the suppressor cell was a macrophage (Sjöberg, 1972). Thus, taken together, the findings of Byfield *et al.* (1973) and Sjöberg (1971, 1972) provided further evidence that in some cases, suppressor macrophages can be generated in response to activated T cells.

In addition to the suppressed humoral responses observed in chronic GVH disease, cellular reactions have also been shown to be suppressed. Lapp and Möller (1969) have shown that skin grafts from allogeneic mice survived significantly longer when grafted to unrelated F_1 hybrid recipients undergoing GVH disease. Both primary and secondary set rejection times could be delayed. However, the mechanism for this immune suppression has not been extensively studied.

Chronic GVH disease then appears to be another example of a situation in which suppressed immune responses can be demonstrated *in vivo* and in which the suppression of the responses *in vitro* can be shown to be mediated by macrophages. Further, as in the case of antigenic competition, it seems likely that the suppressor macrophages are T cell-dependent.

3.4.7. *The Role of Macrophages in Immunosuppression Associated with Cancer and Other Pathological Conditions*

Cancer, miliary tuberculosis, sarcoidosis, lepromatous leprosy and severe viral illnesses are among those diseases associated with depressed humoral and cellular responsiveness (Hersh *et al.*, 1973; Smith, 1972; Turk and Waters, 1971). Patients with advanced cancer have long been known to have suppressed delayed hypersensitivity responses when skin tested with tuberculin (Renaud, 1926), mumps (Logan, 1956; Lamb *et al.*, 1962) and other microbial antigens (Logan, 1956; Lamb *et al.*, 1962; Solowey and Rappaport, 1965). In addition, there is evidence that some cancer patients have a delayed rejection of allogeneic grafts of skin and tumor cells (Gardner and Preston, 1962). Lee *et al.* (1970) were able to demonstrate significant impairment of the ability of non-lymphoid cancer patients to generate serum levels of antibody to flagellin from *Salmonella adelaide* when compared to the response of age-matched, hospitalized patients with diseases thought not to have immunosuppressive effects. Whether such depressed cellular and humoral immune responses precede and perhaps play an etiologic role in these conditions, or are a result of them is uncertain. In the case of cancer patients, however, the demonstration of an anergic state is frequently associated with a poor prognosis (reviewed by Hersh *et al.*, 1973). Thus, insight into the mechanisms resulting in such disease-related immunosuppression could be helpful in developing a more sophisticated approach to the medical management of this group of patients.

Patients with multiple myeloma frequently have suppressed serum levels of normal

immunoglobulin, respond poorly to antigenic stimulation, and are highly susceptible to infection (Fahey *et al.*, 1963; Broder *et al.*, 1975). Broder *et al.* (1975) have shown that PBL from multiple myeloma patients cultured *in vitro* with pokeweed mitogen (PWM) synthesized significantly less immunoglobulin than PBL from normal volunteers under similar conditions. In addition, in many cases, PBL from multiple myeloma patients, when added to normal PBL, suppressed their immunoglobulin synthesis in response to PWM. When phagocytic mononuclear cells were depleted from the PBL of the multiple myeloma patients, the ability to suppress normal PBL immunoglobulin synthesis in response to PWM was largely abrogated. Moreover, the response of PBLs of a myeloma patient to PWM was greatly improved by removal of phagocytic cells. PWM induces a blastogenic response in both T cells and B cells (reviewed by Oppenheim and Rosenstreich, 1976) and X-irradiation of human B cells prior to culture with PWM prevents the appearance of immunoglobulin in the media (T. Waldmann, personal communication). These findings suggest that monocytes in the PBL of some myeloma patients can suppress the proliferation and immunoglobulin production of human B cells *in vitro*. Similarly, in a rodent plasmacytoma model (Krakauer *et al.*, 1976), ascitic fluid from tumor-bearing hypogammaglobulinemic mice was found to contain a low molecular weight trypsin-sensitive factor which suppressed PWM-induced immunoglobulin synthesis by normal mouse spleen cells or human PBL. When the ascites from such mice was depleted of phagocytic cells, the suppressor factor could no longer be demonstrated. Similarly, Kolb *et al.* (1977) have found that macrophages in the spleens taken from hypogammaglobulinemic mice bearing large plasmacytomas suppressed the PFC response of normal spleen cells to SRBC in a Mishell-Dutton culture system when added in as third parties. These studies taken together provide evidence that in both mice and humans suppressor macrophages and/or their products can suppress the production of immunoglobulin by B cells from normal or tumor-bearing individuals *in vitro*. These findings are consistent with the findings of Kirchner *et al.* (1976) and others (Yoshinaga *et al.*, 1972) that in animal systems, B cell mitogen-induced proliferative responses can be suppressed by suppressor macrophages.

Allogeneic skin graft survival was prolonged on some patients bearing malignant neoplasms (Gardner and Preston, 1962). A number of *in vitro* observations of suppressed MLC responses using cells from cancer patients have provided evidence that suppressor macrophages may be involved in this form of immunosuppression also. Berlinger *et al.* (1976) have compared MLC reactivity of PBL from patients with solid, non-lymphoid malignancies to the reactivity of PBL from normal donors. They were able to identify a group of cancer patients in whom MLC reactivity was consistently depressed compared to normal controls. When the PBL with suppressed responsiveness were passed through a Sephadex G-10 column which selectively depleted monocytes, the MLC reactivity was improved in all cases which were suppressed. When PBL from normal donors were similarly filtered, no such effect was seen. Our laboratory (Jerrells, Dean and Herberman, unpublished observations) has confirmed that the lymphoproliferative responses of some cancer patients to mitogens or to allogeneic PBL could be significantly increased by passage of their PBL over Sephadex G-10 columns. Furthermore, the addition of mitomycin C-treated PBL from these patients to cultures of normal PBL could significantly inhibit proliferative responses to T cell mitogens. However, this mechanism does not appear to account for the depressed lymphoproliferative responses of all such patients, since in some cases, the responses remained depressed after removal of adherent cells. Twomey *et al.* (1975) have shown that PBL from patients with Hodgkin's disease stimulated normal PBL poorly in MLCs. When adherent cells were depleted from the patients' cells used as stimulators, strong MLC responses were observed. They could show that the suppressor cell was radioresistant and required protein synthesis to be suppressive, but their failure to show the cell was phagocytic using a 'modified technique' led them to postulate that the suppressor cell was a lymphocyte similar to that observed in normal rat spleen cells by Folch and Waksman (1974). As discussed

earlier, recent evidence suggests that the cells described by Folch and Waksman were actually T cell-dependent suppressor macrophages. Thus it is possible that the findings of Twomey *et al.* (1975) may also support the notion that suppressor macrophages from PBL of cancer patients can suppress the proliferative phase of the response to alloantigens *in vitro*.

Studies in experimental animal tumor systems have provided a firm basis for the role of suppressor macrophages in the immunodepression of tumor-bearing individuals. Our laboratory (Fernbach *et al.*, 1976b) has shown that spleen cells from mice bearing MSV-induced tumors responded poorly in MLC and CML reactions. As described earlier, these depressed responses were shown to be mediated by suppressor macrophages. Veit and Feldman (1976a) have studied a rat tumor model in which they found suppressed MLC responsiveness with spleen cells from tumor-bearing rats. Extensive cell characterization procedures identified the suppressor cells as macrophages (Veit and Feldman, 1976b). Eggers and Wunderlich (1975) have provided evidence that spleen cells from mice bearing a methylcholanthrene-induced tumor also contained suppressor macrophages which suppressed the *in vitro* generation of cytotoxic effector cells to alloantigens. In contrast, Fujimoto *et al.* (1976a) have reported that when mice bearing methylcholanthrene-induced tumors served as donors of thymocytes to be adoptively transferred to syngeneic mice just prior to tumor inoculation, the rate of eventual tumor regression was slowed. Since thymocytes treated with anti-theta and complement had no such effect, Fujimoto *et al.* (1976b) concluded that the suppressor cells were T cells. However, since survival rates of the 'suppressed' and the nonsuppressed groups were identical, despite significant ($p < 0.001$) differences in the rate of tumor regression, it is difficult to draw firm conclusions from these experiments. Further, since all of the experiments were performed *in vivo*, the mechanism of the enhanced tumor growth, i.e. whether mediated through suppressor macrophages and acting on the immune response to the tumor or by direct enhancement of the growth of the tumor, would seem to be an open question.

Thus, suppressed T cell responses to alloantigens have been demonstrated in a variety of tumor systems. In mice, rats and humans, suppressed MLC responses have been shown to be due to cells which have been characterized as monocytes or macrophages by a number of criteria. Spontaneously occurring tumors as well as tumors induced by virus infection and carcinogen inoculation have been shown to induce suppressor monocytes or macrophages in the PBL of humans or spleen of mice and rats which can suppress reactivity to alloantigens. Therefore, these *in vitro* findings provide at least one possible explanation for the observations that allogeneic skin graft survival is prolonged in cancer patients. It would seem that further investigations designed to clearly define the role of suppressor macrophages in suppression of immune responses to alloantigens *in vivo* are indicated.

In addition to cancer, certain infectious diseases are known to be associated with suppression of immune responses. Generally, the degree of immunosuppression has correlated with the severity of the disease. The cause and effect relationship between the phenomena is an important question to be answered. Leprosy, a human disease caused by infection with *Mycobacterium leprae*, has provided insight into the correlation of immunosuppression with the severity of disease. Turk and Waters (1971) studied histological sections of lymph nodes taken from patients with leprosy varying in severity from *tuberculoid* leprosy (with the best prognosis), through *borderline* leprosy (an intermediate stage), to *lepromatous* leprosy (disseminated disease). *Tuberculoid* leprosy was associated either with a completely normal appearing lymph node or with nodes demonstrating a lymphocytosis of the paracortical areas [generally considered to be thymus-dependent (Parrot and Desousa, 1971)]. Occasionally, lymphoblasts were noted in the paracortical areas, indicating the presence of ongoing lymphoproliferative responses. These patients responded well to intradermal lepromin testing and could be sensitized to dinitrochlorobenzene (DNCB). Lymph nodes from patients with leprosy of intermediate severity showed an infiltration of the paracorti-

cal regions with epithelioid-like cells which remained distinctly separate from the lymphocytes. These patients responded to intradermal lepromin albeit poorly, but could be sensitized to DNCB. In *lepromatous* leprosy, paracortical areas of lymph nodes were heavily infiltrated with foamy histiocytes and the germinal centers manifested a hyperplastic plasmacytosis. These patients had a negative lepromin test and usually could not be sensitized to DNCB. Thus, this study provides *in vivo* morphological evidence for an association between severity of immunosuppression and macrophage infiltration of lymphoid tissues.

3.5. THE DIFFERENTIAL EFFECT OF MACROPHAGES ON NORMAL VERSUS NEOPLASTIC CELLS

Keller compared the effects of activated macrophages on normal versus neoplastic cells and found that, although both cell types undergo inhibition of proliferation, only neoplastic cells were killed (Keller, 1974a, 1976). Evidence supporting the notion that only neoplastic cells are killed by macrophages, thus establishing the concept of nonspecific but 'selective' macrophage-mediated cytotoxicity, has been provided by several laboratories (Currie and Basham, 1975; Cleveland *et al.*, 1974; Hibbs, 1973; Holterman *et al.*, 1973; Hibbs *et al.*, 1973; Piessens *et al.*, 1975). All of these studies, however, measured target cell lysis rather than target cell proliferation. Our laboratory (Kirchner *et al.*, 1974a, b, 1975) has noted on several occasions that the suppressive effect of macrophages on lymphocytes was not observed unless the lymphocytes were proliferating rapidly (i.e. stimulated by mitogens, tumor antigens, or alloantigens, etc.). One might therefore speculate that some property of transformed cells which is shared by proliferating lymphocytes but not by resting lymphocytes provides the basis by which macrophages exert their cytolytic and/or cytostatic effect. Evidence suggesting that rapidly proliferating cells are more susceptible to lysis by macrophages than resting cells has been provided by Holterman *et al.* (1975). They have shown that rat embryo fibroblasts are susceptible to cytolytic effects only during early passages in tissue culture, when their proliferation rate is high. They hypothesized that neoplastic cells may have certain membrane properties in common with rapidly proliferating embryonic cells of early passage *in vitro* which are subsequently lost through the process of differentiation. Fox *et al.* (1971) have provided evidence that mild proteolytic treatment of normal mouse fibroblasts maintained *in vitro* (3T3) resulted in the appearance of certain agglutinin receptors on the surface of the cell membrane which could always be demonstrated on the membrane of polyoma virus transformed mouse fibroblasts (Py3T3). The expression of the agglutinin receptors on the 3T3 cells was associated with an increased rate of proliferation and a loss of contact inhibition. The effect was transient, however, and when the proliferation rate returned to normal, the agglutinin receptors were no longer demonstrable. Although these studies were done with fibroblasts and not with lymphoid cells, they at least support the notion that rapidly proliferating cells may express receptors or membrane properties normally expressed 'selectively' on continually proliferating (transformed) cells. Shinitzky and Inbar (1974) have provided evidence that the microviscosity of the surface membrane lipid layer of normal lymphocytes and malignant lymphoma cells can be associated with the growth characteristics of the cells, and that by altering the microviscosity of the membrane with lecithin-cholesterol (1:1) liposomes, one can predictably modify the growth characteristics of the cells. These observations further emphasize the possibility that normal cells may transiently acquire membrane characteristics of transformed cells during blastogenesis. Thus, if the mechanism by which macrophages lyse transformed cells is the same as that mediating cytostasis of both cell types, the ability of lymphocytes to cover or fail to express some membrane property when their proliferation is inhibited could account for the findings that macrophages have cytostatic effects on both cell types but lyse only cells which are transformed. Another entirely possible explanation for the findings is that the effector cells involved in the cytotoxicity are not macrophages at all but instead are natural killer (NK) cells as recently described by Herberman *et*

al. (1975). Recently Herberman *et al.* (1977) have shown that many agents with well-known RES activating effects strongly boost NK cell activity when administered to mice, and that the broad specificity of the cytolytic activity is directed primarily toward tumor cells. Similarly, Wolf *et al.* (1976) observed that i.p. injection of BCG caused the appearance of increased cytolytic activity by PEC and that most, if not all, of this activity was mediated by NK cells. Our laboratory is currently investigating the possibility that at least some of the selective cytolytic effects of PEC previously attributed to macrophages may be mediated by highly active NK cells.

4. CONCLUDING REMARKS

In this review we have discussed some of the evidence that macrophages provide both enhancing and suppressive effects on immune responses. The enhancing effects of macrophages might be grouped into three broad categories: prevention of tolerance, antigen presentation and enhancing factor production. In the case of subcellular protein antigens, macrophages phagocytize and break down much of the antigen, thus helping to prevent a prolonged interaction of B cells with high concentrations of free soluble antigen, in the absence of a second signal to produce antibody, which would be likely to result in a state of B cell tolerance. Some of the antigen, however, is not broken down by macrophages, but instead is expressed on the cell surface in a highly immunogenic form. Recent evidence suggests that one form taken by such antigen is that of a complex with the MHC antigens on the surface of the macrophage. As such, at least T cells can be primed or triggered with the resultant generation of helper T cells to provide a second signal to B cells for antibody production and/or a clone of highly reactive memory T cells which are then available to mediate a vigorous secondary response when presented with the same antigen in complex with the same MHC determinant on the surface of a macrophage. In addition to presentation of soluble antigens to T cells, there is a wealth of information suggesting that macrophages produce factors which can enhance the response of T cells to mitogens as well as to cellular antigens such as alloantigens in MLC or antigens on the surface of heterologous erythrocytes. More detailed discussion of these factors can be found elsewhere in this series. Less is known about the macrophage-B cell interaction. There are a number of reports showing that macrophages can stimulate B cells directly to proliferate and produce antibody. In addition, there is some evidence that macrophages may play a vital role in mediating T cell-B cell collaboration. Further investigations are needed to clarify these issues.

On the other hand, we have presented evidence that macrophages, if in sufficient quantity or if sufficiently activated, can suppress immune responses which are dependent on the expansion of a clone of sensitized lymphocytes. Suppression of T cell proliferation by suppressor macrophages *in vitro* can be correlated with evidence obtained *in vivo* indicating that when macrophages were strongly activated during the time when a lymphocyte proliferative response should have occurred, the resultant immune state was frequently depressed. Similarly, since suppressor macrophages could also suppress B cell proliferative responses *in vitro*, it is not surprising that antibody responses requiring B cell proliferation were frequently suppressed in cases where the RES was highly activated such as in chronic graft versus host disease, some cases of antigenic competition and in the presence of a rapidly growing malignant tumor.

The suppressive effect of macrophages would seem to be important for several reasons. We have discussed evidence that suppressor macrophages could be induced by lymphocyte mediators. The best studied mediator apparently capable of inducing suppressor macrophages is MIF. In fact, as discussed, it seems likely that many if not all suppressor T cells may liberate factors which mediate the suppressor effect through macrophages. This then would appear to represent a mechanism by which a clonal expansion of sensitized lymphocytes could be slowed or stopped by a product of lymphocyte activation. Since 2 or 3 days are required to activate macrophages with

lymphocyte mediators (see Section 3.2.4.), such a mechanism would not prevent the appearance of a significant number of effector cells and memory cells but could serve to limit their continued, excessive production. Such negative feedback systems have been described for many regulatory mechanisms in mammalian physiology. As we have discussed, suppressor macrophages have little or no suppressor effect on cytotoxic T cells or other effector cells which do not proliferate. Thus, established immune responses would not be affected through this mechanism.

In addition, macrophages can be activated to become suppressive by agents which may operate through mechanisms independent of lymphocytes. Agents or conditions which activate the RES, including the growth of many tumors, have frequently resulted in the appearance of splenic suppressor macrophages. Although in this review we have concentrated on reports of immunosuppression associated with administration of RES activators (*C. parvum* and BCG), in fact, these agents are generally thought of as immunopotentiators. However, a problem with the use of such agents is that the immunopotentiating effects are seen inconsistently and, frequently, immunosuppression is seen instead. The effects of these agents on the immune response *in vivo* are undoubtedly complex. When used correctly, RES stimulators may eventually prove very useful for immunotherapy. However, when immunosuppression occurs instead of immunopotentialiation, it seems likely that suppressor macrophages play a role in the suppression. In the case of RES activation in response to tumor growth, an interesting situation arises. Although perhaps an appropriate response to the tumor, if the RES activation cannot control the growing neoplasm by direct cytostatic effects on the tumor cells, it is entirely possible that the resulting abundance of suppressor macrophages may play a role in the immunosuppressed state frequently observed in cancer patients. There are undoubtedly many ways in which tumors can cause immunosuppression. One way, however, might be through a mechanism by which tumor cells or tumor cell products lead to an activated RES which suppresses immune responses requiring lymphocyte proliferation. In this case, however, unlike the response of local macrophages to mediators from sensitized, activated, proliferating lymphocytes, the RES stimulus from the tumor might be more diffuse, involving a significant proportion of the lymphoid cells. One would predict that this would result in a prolonged, nonspecific state of immunosuppression.

Finally, we have briefly discussed the apparently reproducible observations that, although the cytostatic effects of macrophages on rapidly dividing lymphocytes and transformed cells seem highly correlative, the 'lytic effects of macrophages' are directed selectively toward cells that are neoplastic. Assuming that macrophages are indeed the effector cells, we have suggested that the basic recognition process leading to the cytostatic and the cytolytic events are similar, and that the selective lysis of neoplastic cells reflects the inability of tumor cells to modulate certain surface receptors shared by normal lymphocytes only during blastogenesis.

In summary, macrophages seem to be involved in almost every phase of the immune response. They can both enhance and suppress lymphocyte responses, and most lymphocyte responses require the presence of macrophages for any of a number of reasons to mount any response at all. The observations that suppressor T cells may mediate some if not all of their suppressor effects through suppressor macrophages help to explain what appeared to be a dual suppressor mechanism triggered by similar stimuli but mediated by different effector cells. The evidence discussed in this review supports the concept that macrophages play a central role in regulating some aspects of the immune response. Hopefully, further investigations of the immunobiology of macrophages will enable careful therapeutic intervention via this natural regulatory mechanism.

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