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Regulation of Human B Lymphocyte Activation, Proliferation, and Differentiation

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I. Introduction

The induction of an antibody response is a complex process that at a cellular level involves triggering of B cell precursors to become high-efficiency antibody-secreting plasma cells. On the basis of work by a number of investigators, a model of this process has been developed (Fig. 1) in which initial activation of resting B cells by antigen is followed by proliferation of the activated precursors and, finally, differentiation of some of the resultant daughter cells into mature nondividing antibody-secreting cells (Dutton, 1975; Schimpl *et al.*, 1974). These stages of the development of B cell precursors to antibody-secreting cells have been viewed as sequential events that are initiated and regulated by distinct and specific signals.

The basis for this model initially was derived from the experiments of Schimpl and Wecker (1972), Hunig *et al.* (1974), Dutton (1975), and Parker (1975), demonstrating that antigen or its surrogate, anti-immunoglobulin (anti-Ig), stimulated B cell activation and DNA synthesis; T cell-derived lymphokines were required to promote the subsequent differentiation of postdivisional daughter cells into antibody-secreting cells. Data of this nature suggested that antigen alone stimulated activation and clonal expansion of the B cell precursors which was then followed by terminal differentiation induced by T cell help. More recent data have suggested that regulation of B cell responsiveness may be far more complicated, with a variety of individual signals, many of T cell origin, playing a role in the activation and proliferation of B cell precursors as well as their differentiation into immunoglobulin-secreting plasma cells (reviewed by Vitetta *et al.*, 1984a; Howard *et al.*, 1984; Kishimoto, 1985).

One of the initial events involved in B cell activation is thought to be the interaction of surface Ig with antigen (Bretscher and Cohn, 1970). Depending on the nature of the ligand interacting with surface Ig and the phenotype of the responding B cell, additional signals may

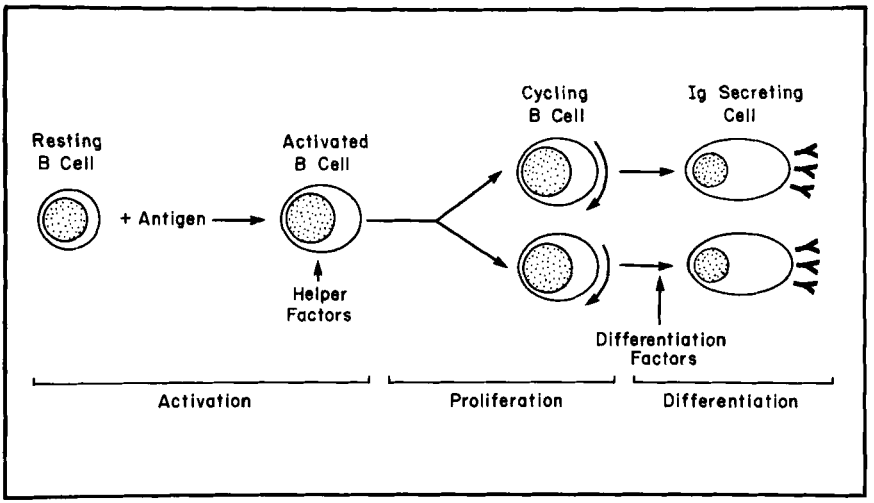

FIG. 1. Model of B cell activation, proliferation, and differentiation.

be involved in the subsequent differentiation of the activated B cell into an antibody-secreting cell. Especially important is the influence of T cells on B cell activation, proliferation, and differentiation. Although initially it was felt that only a limited number of T cell products were involved and that T cell influences were critical largely for differentiation after activation and proliferation (Dutton, 1975; Schimpl *et al.*, 1974; Schimpl and Wecker, 1972; Hunig *et al.*, 1974; Parker, 1975), more recent evidence suggests that a variety of T cell-derived lymphokines may potentially be involved in each step of B cell responses. Indeed, it appears likely that a T cell lymphokine may even play a role in preparing small resting B cells to respond before engagement of surface Ig receptors (Oliver *et al.*, 1985; Rabin *et al.*, 1985; Bowen *et al.*, 1986).

B cell responses induced by various stimuli may be discriminated by the apparent need for T cell influences to support the differentiation of antibody-forming cells. Classically, B cell responses have been defined as T cell independent (TI) and T cell dependent (TD). A number of common properties have been described that are useful in distinguishing TI antigens from TD antigens. For example, most TI antigens are poorly metabolized high-molecular-weight polymers with simple repeating structures (Britton *et al.*, 1968; Sela *et al.*, 1972). These properties have been postulated to be important in inducing cross linking of cell surface receptors, thus providing a maximal activation signal (reviewed by Mosier and Subbarao, 1982). The T

cell independence of a variety of stimuli in the murine system, e.g., polymerized flagellin (Armstrong *et al.*, 1969; Feldmann and Basten, 1971), lipopolysaccharide (LPS; Moller and Michael, 1971; Kearney and Lawton, 1975), pneumococcal polysaccharide (Howard *et al.*, 1971), haptenated or nonhaptenated *Brucella abortus* organisms (Mond *et al.*, 1974), polyacrylamide (Feldmann *et al.*, 1974), ficoll (Mosier *et al.*, 1974), dextran (Dorries *et al.*, 1974), or levan (Miranda, 1972), has been well established. Many T1 antigens such as LPS also function as polyclonal B cell activators.

TI responses have been further subdivided into two classes on the basis of the ability of TI stimuli to activate B cells from the CBA/N mouse strain which lacks the Lyb5 positive subset of mature B lymphocytes (Ahmed *et al.*, 1977). Thus, TI-1 stimuli such as LPS can stimulate B cells from CBA/N mice (Mond *et al.*, 1978a) whereas TI-2 stimuli such as ficoll and dextran do not (reviewed by Mosier *et al.*, 1977a).

Few agents have convincingly been shown to induce the differentiation of Ig-secreting cells (ISC) in man without T cell influences. Moreover, a delineation of TI-1 and TI-2 responses has not been accomplished in human studies. *Nocardia* water-soluble mitogen (NWSM; Waldmann and Broder, 1982), *Salmonella paratyphi* B (Chen *et al.*, 1981), and *Klebsiella pneumoniae* (Gross and Rucks, 1983) have been suggested to function as TI stimuli in man. It is important to note, however, that responsiveness to NWSM has been shown to be considerably enhanced by T cells (Waldmann and Broder, 1982) and inconsistent results have been obtained when responses to *Salmonella* organisms have been examined in detail (Jelinek and Lipsky, 1983). In our hands, all polyclonal B cell activators with the exception of Epstein–Barr virus (EBV) have been found to require T cells or T cell-derived factors for the generation of Ig-secreting cells in man, although some will induce B cell proliferation in a T cell-independent manner (see below). In this regard, several recent studies in the murine system have suggested that T cell influences play a role in the activation of B cells by TI antigens and thus have challenged the notion that B cell responsiveness occurs in a truly T cell-independent fashion (Endres *et al.*, 1983; Mond *et al.*, 1983; Thompson *et al.*, 1984).

TD antigens have been defined as those antigens requiring T cell help to permit the stimulation of B cell differentiation into antibody-secreting cells. T cell-dependent responses include both antigenic and mitogenic responses, each of which can be further divided into two subgroups. The first group includes responses in which physical

interactions between the B cell and T cell must take place whereas the second requires only the influence of T cell-derived lymphokines. Work in both the human and murine systems has demonstrated that small resting B cells are poorly responsive to nonspecific T cell-derived soluble factors in that such factors usually do not directly stimulate proliferation and differentiation (Andersson *et al.*, 1980; Principato *et al.*, 1983). An initial physical interaction with T cells appears to be necessary to activate such B cells, after which the cells may acquire direct responsiveness to nonspecific factors (Kuritani and Cooper, 1983; Peters and Fauci, 1983; Chiorazzi *et al.*, 1979; Peters *et al.*, 1985; Howard *et al.*, 1984; Coutinho *et al.*, 1984; Brenner *et al.*, 1984; Noelle *et al.*, 1983). By contrast, other populations of B cells may be stimulated to proliferate and differentiate in the absence of intact T cells when T cell influences are provided only by soluble lymphokines (reviewed by Vitetta *et al.*, 1984a; Howard *et al.*, 1984; Kishimoto, 1985). These results have suggested that the state of activation of the B cell subpopulation under study determines the nature of the interaction with T cells required for the initiation of T cell-dependent B cell responses. Thus, it has been suggested that "resting" B cells may require physical interactions with T cells whereas "activated" B cells may respond in the presence of soluble T cell factors. These contentions, however, have recently been challenged by several investigators. The findings of Mosier (1986) indicated that both large and small antigen-specific murine B cells required an MHC-restricted T–B cell interaction to secrete specific antibody. Moreover, in man T cell-derived lymphokines have been shown to support responsiveness of both small resting and activated B cells after appropriate stimulation (Jelinek *et al.*, 1986a). In addition, the concept that T cell-derived helper factors are not active on resting B cells has been challenged. In the murine system, Oliver and co-workers (1985) and Rabin and colleagues (1985) have shown that B cell stimulatory factor-1 (BSF-1; formerly referred to as B cell growth factor-I, or BCGF-I) stimulates small resting B cells in the absence of intact T cells. Therefore, the concept that the activation status of the B cell is the only determinant of the signals necessary for stimulation appears to be unlikely.

A variety of surface markers have also been used to delineate responsive B cell subsets (Janossy and Greaves, 1975; Ahmed and Smith, 1982, 1983). Expression of cell surface Ig isotypes has proved to be useful in this regard (reviewed by Finkelman and Lipsky, 1979; Vitetta *et al.*, 1980). Delineation of responsive subsets by virtue of cell surface IgD (sIgD) has been particularly useful. Expression of sIgD

varies during B cell maturation, activation, and differentiation. Immature resting B cells, activated mature B cells, and post-switch-memory B cells all exhibit decreased sIgD compared to mature resting B cells that express both cell surface IgD and IgM (Abney *et al.*, 1978; Sitia *et al.*, 1979; Coffman and Cohn, 1977; Black *et al.*, 1978, 1980; Preud'homme, 1977; Monroe *et al.*, 1983). Expression of sIgD by B cells can be related to the responsiveness of the population to various stimuli (Gronowicz *et al.*, 1979; Kuritani and Cooper, 1982; Saiki and Ralph, 1982; Jelinek *et al.*, 1986a). Study of B cell subsets defined on the basis of other surface Ig isotypes has also been useful in delineating functional subsets and in monitoring isotype switching during immune responses (Abney *et al.*, 1978; Kanowith-Klein *et al.*, 1981; Benner *et al.*, 1981; Teale *et al.*, 1981).

Additionally, non-Ig surface markers have also been used to delineate the functional heterogeneity of B cells. These include the Lyb5 marker in the murine system (Huber *et al.*, 1977; Ahmed *et al.*, 1977; Mond *et al.*, 1978; Mosier *et al.*, 1977b; Singer *et al.*, 1981), the B1 antigen found on human B cells (Stashenko *et al.*, 1980; Nadler *et al.*, 1981a; Boyd *et al.*, 1985a; Anderson *et al.*, 1985), and complement receptors found on both murine and human B cells (Lindsten *et al.*, 1985; Nemerow *et al.*, 1985a).

Much of what is currently understood about human B cell biology has been primarily gained through studies employing various mitogens rather than specific antigens. The use of antigen-specific B cell systems has been limited by the low precursor frequency of responding cells. However, a number of investigators have successfully studied antigen-specific human B cell responses (Hoffman, 1980a; Misiti and Waldmann, 1981; Lane *et al.*, 1981; Kehrl and Fauci, 1983; Peters and Fauci, 1983). These studies have been especially helpful in delineating the characteristics of B cells recently activated by *in vivo* immunization and have shown that these cells are largely refractory to further stimulation by antigen or polyclonal B cell activators but do respond directly to T cell-derived helper factors. However, attempts to study primary B cell responses to specific antigens have met with considerable difficulty. Therefore, an understanding of the activation of resting human B cells has largely derived from studies employing various polyclonal B cell activators.

In this article, several specific aspects of human B cell activation will be reviewed. The sequence of events and the signals involved in initial B cell activation and the relationship to subsequent proliferation and the generation of Ig-secreting cells will be delineated. The functional and phenotypic heterogeneity found among B cells as well

as activation requirements of specific B cell subpopulations will be examined. Finally, the roles of various cytokines including B cell growth factor (BCGF), interleukin 2 (IL-2), interferon-gamma (IFN-γ), and interleukin 1 (IL-1) in B cell responses and their temporal relationship to other B cell activation events will be reviewed. The goal of this review article is the development of a testable model of the generation of antibody-forming cells from human B cell precursors.

II. B Cell Proliferation and Antibody Responses

B cell proliferation plays an important role in the generation of humoral immune responses. Proliferation of B cell precursors provides a mechanism whereby a small number of antigen-specific cells can be expanded to permit the generation of an effective antibody response (Burnet, 1959). Beyond the need for clonal expansion of B cell precursors, it has been found that cell division is a necessary step in the differentiation of B cells into mature antibody-secreting cells. Thus, inhibition of B cell proliferation has been shown to prevent the generation of Ig-secreting cells in many model systems. For example, Fauci *et al.* (1978) found that pokeweed mitogen (PWM)-stimulated differentiation of anti-sheep red blood cell (SRBC)-specific antibody-forming cells from human peripheral blood B cells was inhibited by low doses of irradiation, with complete suppression of responses observed after exposure to 300 to 500 rads. Other investigators have utilized cell cycle-specific inhibitors to demonstrate the role of cell division in B cell differentiation. Jelinek and Lipsky (1983) demonstrated that treatment of human peripheral blood B cells with mitomycin C, a specific inhibitor of DNA synthesis, completely prevented the generation of Ig-secreting cells in response to PWM, Cowan I strain *Staphylococcus aureus* (SA), or *Salmonella typhimurium*. These same investigators observed similar results when hydroxyurea (HU), another inhibitor of cellular DNA synthesis (Sinclair, 1965; Krakoff *et al.*, 1968), was employed. Hydroxyurea has also been found to inhibit the generation of SRBC-specific antibody-secreting cells from murine spleen cells (Merrill and Ashman, 1980; Jaroslow and Ortiz-Ortiz, 1971).

These findings indicate that B cell proliferation plays a required role in the differentiation of antibody-secreting cells beyond the requirement to expand the number of antigen-reactive cells. Cellular division appears to play a necessary role in the maturation of precursors of antibody-secreting cells. Evidence from a variety of studies indicates that cell division is important in the differentiation of many

different lineages of cells (reviewed by Holtzer *et al.*, 1972). The precise physiologic and molecular events in the differentiation of Ig-secreting cells that depend upon cellular division remain largely unknown.

Whereas B cell proliferation appears to be necessary for the generation of antibody-forming cells from resting B cells in many model systems, the conclusion that precursor cell division is always a prerequisite for the differentiation of antibody-secreting cells remains a matter of some controversy. Andersson and Melchers (1974) have demonstrated that small resting murine B cells were able to mature to 19 S IgM-secreting cells in the absence of DNA synthesis and proliferation. In addition, Melchers *et al.* (1980) described a factor produced by antigen-activated T cells that stimulated small resting murine B cells in the presence of specific antigen to mature into IgM- and IgG-secreting cells in the absence of cellular division. A number of circumstances have also been described in which the generation of ISC in man may occur without antecedent B cell proliferation. Neckers *et al.* (1985) found that an antibody to the transferrin receptor blocked human peripheral blood B cell proliferation stimulated by SA and a T cell factor and inhibited the total amount of IgM secreted during a 5-day culture period but did not prevent IgM secretion by the cells persisting in the culture. In addition, Chen *et al.* (1981) reported that the polyclonal B cell activator, *Salmonella paratyphi* B, appeared to activate human peripheral blood B cells to produce large amounts of Ig in the absence of any detectable DNA synthesis. Similar conclusions were also reached by Gross and Rucks (1983), who stimulated B cells with *Klebsiella pneumoniae*. Finally, Grayson *et al.* (1981) reported that hydrocortisone stimulated Ig production from human peripheral blood B cells without accompanying cellular proliferation. Because B cell proliferation was only assayed by the incorporation of [^{3}H]thymidine in these studies, it is possible that division of precursor B cells occurred but the number of responding cells was so small that it could not be detected by this technique. In addition, the activation status of B cells responding to these polyclonal activators was not delineated and thus it is not known whether differentiation was stimulated from B cells that had recently divided *in vivo*. In this regard, Muraguchi *et al.* (1981) reported that an EBV-transformed human B lymphoblastoid line (CESS) responded to T cell factors with an increase in IgG-production in the absence of cellular division, suggesting that B cells at a later stage of differentiation may not require cell division before acquiring the ability to secrete Ig. It is thus possible that under certain circumstances a small population of previously acti-

vated or even small resting B cells may be induced to secrete Ig without antecedent division, although it is clear that for most precursors of Ig-secreting cells, cellular division is a prerequisite for differentiation.

In contrast to the detailed examination of the requirement of proliferation in the subsequent differentiation of antibody-secreting cells, fewer studies have addressed the role of ongoing proliferation of Ig-secreting cells in the development of the total antibody response. It has been suggested that proliferation diminishes as differentiation proceeds. Thus, for example, Ralph and Kishimoto (1981) demonstrated a negative correlation between Ig secretion and growth in phorbol myristate acetate (PMA)-stimulated human EBV-transformed B lymphocyte cell lines. Similarly, Primi (1983) found an activity in conditioned medium from a T cell hybridoma that inhibited lipopolysaccharide (LPS)-induced proliferation of murine spleen B cells but supported a high rate of Ig synthesis. These results suggested that differentiation and proliferation were mutually exclusive. By contrast, the studies of Jaroslow and Ortiz-Ortiz (1971) and Merrill and Ashman (1980) suggested that ongoing proliferation of antibody-secreting cells may be necessary for the evolution of antibody responses. These investigators found that HU inhibited the primary *in vitro* response of murine spleen cells to sheep erythrocytes when added at the beginning of culture or later when large numbers of Ig-secreting cells were

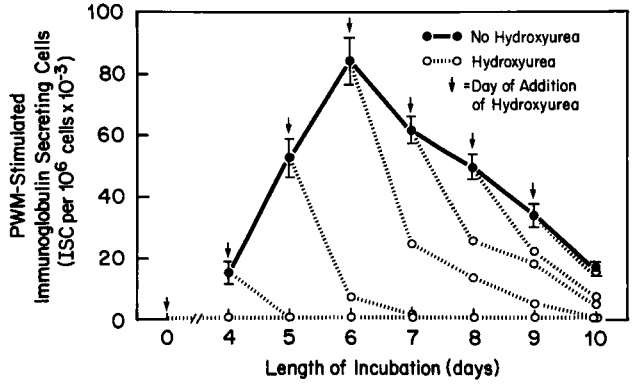

FIG. 2. Role of ongoing B cell proliferation in the continued expression of PWM-stimulated generation of Ig secreting cells (ISC). PBM were stimulated with PWM and assayed for the number of ISC found on days 4 through 10 of culture. HU (10^{-2} *M*) was added at the initiation of culture and on consecutive days thereafter. For each set of cultures, the number of ISC was then assayed on a daily basis. Control cultures consisted of PBM that were incubated in an undisturbed fashion or received saline at the same time the experimental cultures received HU. From Jelinek and Lipsky (1983).

already present. These results suggested that Ig-secreting cells were an actively dividing population but did not exclude the possibility that the antibody-forming cells were short lived and needed to be continuously replenished from a rapidly dividing precursor pool.

The possibility that B cells continue to proliferate after differentiation into Ig-secreting cells was addressed by the studies of Jelinek and Lipsky (1983). Human peripheral blood mononuclear cells were stimulated with PWM and assayed for the generation of Ig-secreting cells on a daily basis. PWM-stimulated Ig-secreting cells were detected in control cultures after a 4-day incubation, reached a maximum on day 6, and still could be detected on day 10 of culture (Fig. 2). The addition of HU at the initiation of culture completely blocked the development of Ig secreting cells throughout the length of the incubation. The addition of HU on days four through eight, when Ig secreting cells were already present, caused a marked reduction in their number within 24 hours. Similar results were noted when Ig synthesizing cells were examined by staining cells for cytoplasmic Ig. HU-resistant Ig synthesizing and secreting cells did not begin to appear in these cultures until day 6 of the incubation. The percentage of HU-resistant Ig-secreting cells increased between days 6 and 8 of culture and complete resistance to HU did not develop until day 9. To confirm that the Ig-secreting cells were actively dividing rather than being continuously replenished from a rapidly dividing precursor pool, experiments were carried out to analyze the DNA content of individual Ig-synthesizing cells. By simultaneously staining cells for DNA content with ethidium bromide and cytoplasmic Ig it was found that many PWM-stimulated Ig-synthesizing cells were cycling as evidenced by a DNA content characteristic of cells in the S, G_2, or M phases of the cell cycle (Jelinek and Lipsky, 1983). These results support the conclusion that Ig-synthesizing and -secreting cells are an actively proliferating population.

These results indicated that B cells not only require antecedent cell division to differentiate but also suggested that ongoing Ig synthesis and secretion required continued proliferation of the differentiated cells. Moreover, the expansion in the number of ISC detected as the culture was prolonged appeared to require the ISC themselves to undergo continued proliferation. These results and those obtained by other investigators in the murine system (Makela and Nossal, 1962; Perkins *et al.*, 1969; Merrill and Ashman, 1980) supported the conclusion that early in culture Ig-secreting cells are not terminally differentiated cells but rather cells capable of both continued proliferation and Ig secretion.

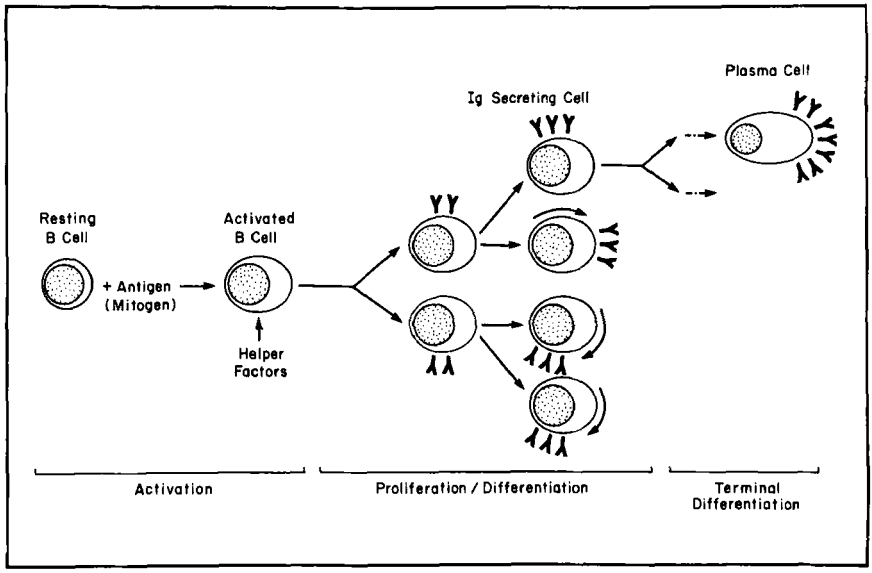

FIG. 3. Role of initial and ongoing proliferation in the differentiation of antibody secreting cells.

The model shown in Fig. 3 incorporates these results in depicting the relationship between B cell proliferation and differentiation into mature antibody-secreting cells. It is important to emphasize that the major difference between this model and the one shown in Fig. 1 is that differentiation is shown to occur during proliferation as an ongoing proliferation-requiring process rather than as a single step. The finding that early in culture Ig-secreting cells are not a stable terminally differentiated population, but rather an actively cycling one, indicates that proliferation of the Ig-secreting cells themselves plays an important role in propagating the antibody response and determining its overall magnitude. In addition, the results imply that factors that alter the continued proliferation of Ig-secreting cells after initial differentiation can affect the magnitude of the immune response. The reasons the cells undergo multiple rounds of division following acquisition of the ability to secrete Ig but before terminal differentiation into an end-stage nondividing plasma cell are not clear. However, it is known that the rate of Ig synthesis and secretion markedly increases after multiple rounds of cell division (Jelinek and Lipsky, unpublished observation). In addition, continued proliferation of the Ig-secreting cells may be important in promoting heavy chain isotype switching (reviewed by Cebra *et al.*, 1984) and providing the opportu-

nity for somatic mutations that increase the affinity of the secreted antibody (reviewed by Milstein, 1986).

Implicit in the model displayed in Fig. 1 is the assumption that the initial proliferative phase is necessary to expand the number of Ig-secreting cell precursors. To test this assumption, Jelinek and Lipsky (1985) stimulated peripheral blood B cells with SA, a polyclonal B cell activator that triggers proliferation but not differentiation into Ig-secreting cells. Addition of T cell factors supports the differentiation of Ig-secreting cells. Moreover, differentiation occurs even when cells are initially preactivated with SA alone for 48 hours and then exposed to T cell factors (Jelinek and Lipsky, 1985). Addition of HU during the initial preincubation with SA eliminated all cells traversing the G_1–S interphase and demonstrated that all the precursors of Ig-secreting cells capable of differentiating in response to SA and T cell factors resided within the population of peripheral blood B cells that were activated to proliferate by SA alone. To determine whether initial B cell proliferation expanded the precursors of Ig-secreting cells, B cells were cultured with SA alone for various lengths of time, harvested, and recultured in the presence of T cell factors (Fig. 4). When the

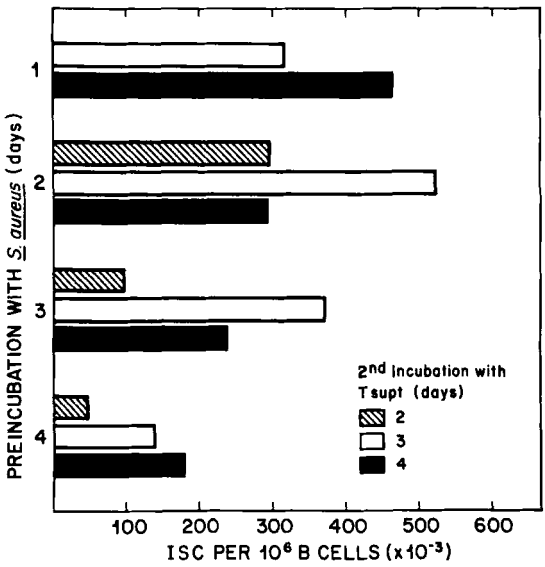

FIG. 4. Effect of preincubation with SA on the expansion of the precursors of Ig secreting cells. B cells were cultured with SA for varying lengths of time (1 to 4 days). Cells were then washed and recultured in the presence of T cell supernatant. Cells were assayed for the generation of Ig secreting cells after 2 to 4 days of the second incubation. From Jelinek and Lipsky (1985).

number of Ig-secreting cells generated was examined after the second incubation, it was found that the precursor pool of Ig-secreting cell progenitors did not appear to be preferentially expanded as the SA-stimulated culture was prolonged beyond 24 hours. This observation indicated that the precursors of Ig-secreting cells are not selectively expanded as a result of SA-induced proliferation and therefore supported the conclusion that in this model system proliferation is necessary to induce responsiveness to differentiative influences but not to increase the size of the responsive precursor pool.

III. Induction of B Cell Responses

Much of our current understanding of human B cell responsiveness has derived from studies using T cell-dependent polyclonal B cell activators (PBA). These include PWM (Janossy and Greaves, 1975; Keightley *et al.*, 1976) and soluble staphylococcal protein A (SPA) (Sjoquist *et al.*, 1972; Lipsky, 1980). Both B cell proliferation and the generation of antibody-secreting cells stimulated by these PBA require T cell help. In addition, anti-immunoglobulin antibodies (anti-Ig) and SA are also T cell dependent but represent a different class of stimuli in that initial activation and DNA synthesis are T cell independent whereas perpetuation of the proliferative response and the generation of Ig-secreting cells are T cell dependent (Sell *et al.*, 1965; Kishimoto and Ishizaka, 1975; Parker *et al.*, 1979; Romagnani *et al.*, 1981; Muraguchi and Fauci, 1982; Jelinek and Lipsky, 1985). In man, anti-Ig and SA differ in that SA is a potent stimulus for the generation of Ig-secreting cells but anti-Ig is not. Since soluble T cell-derived factors can support SA-activated human B cell proliferation and differentiation into Ig-secreting cells very effectively, this PBA has proved useful for studying the signals involved in the regulation of human B cell responses.

Although T cell help may be provided by soluble factors in a number of systems, some B cell responses are initiated only through a direct physical interaction between T cells and B cells (reviewed by Singer and Hodes, 1983). Antigen-specific or linked recognition requires the appropriate hapten-carrier conjugate to stimulate the hapten-specific B cell and the carrier-specific T cell simultaneously. At one time it was thought that effective T–B cell collaboration involved a direct physical interaction between the T cell and the B cell via an antigen bridge (Mitchison, 1971; reviewed by Singer and Hodes, 1983). More recent studies, however, have documented the ability of B cells to serve as antigen-presenting cells (Chestnut and Grey, 1981;

Rock *et al.*, 1984; Tony and Parker, 1985; Lanzavecchia, 1985; Abbas *et al.*, 1985). The requirements for hapten-carrier linkage and MHC-restricted physical interactions therefore appear to be accounted for by the ability of the B cell itself to take up and degrade antigen and present the relevant immunogenic moieties to T cells in the context of self-MHC molecules (Tony and Parker, 1985; Chestnut and Grey, 1981; Rock *et al.*, 1984; Mosier, 1986).

Andersson *et al.* (1980) found that antigen nonspecific factors were incapable of driving growth and Ig secretion of resting murine B cells. However, such nonspecific factors were able to support responses following an MHC-restricted antigen-specific physical interaction between helper T cells and B cells. Similar findings have been obtained in a human system by Principato *et al.* (1983), who demonstrated that activation of small peripheral blood B cells by a TNP-specific cloned T cell line required direct physical interaction between the T cell line and TNP-modified responding B cells. Activation by the PBA, PWM may also involve physical interaction between T cells and responding B cells, although such interactions are neither antigen specific nor MHC restricted. Generation of Ig-secreting cells in response to PWM is completely T cell dependent but poorly supported by soluble T cell factors (Fauci *et al.*, 1976; Suzuki *et al.*, 1986), suggesting that direct T cell–B cell interaction is necessary. Although physical interactions between T cells and B cells may be required in these two human systems, in neither case is the antibody response specific, but rather in each, polyclonal B cell activation is stimulated. Thus, these systems may not be typical examples of linked recognition.

Utilization of B cell responses that can be supported by T cell-derived soluble factors permits a detailed examination of the signals involved in B cell activation, proliferation, and differentiation. Investigators utilizing T cell-dependent B cell systems supported by soluble T cell- and monocyte-derived factors have often used anti-Ig or SA as the initial signal. In a murine system, Howard and Paul (1983) have suggested that a series of exogenous signals may be necessary for anti-Ig-stimulated B cells to progress through the initial cell cycle. Thus, anti-Ig was found to stimulate resting B cells to enter early G_1 where they acquired responsiveness to BCGF. BCGF was found to be necessary to drive the cells into late G_1, at which point they required a mononuclear phagocyte (Mϕ) factor, interleukin 1 (IL-1) to enter the S phase (DeFranco *et al.*, 1982a; Howard *et al.*, 1983). Work by Hoffman (1980b), however, has suggested that the requirement for IL-1 may precede the requirement for BCGF. Moreover, work by Lipsky *et al.* (1983) demonstrated that PWM-stimulated human peripheral blood B

cell differentiation into Ig-secreting cells was inhibited by an antibody to IL-1, but only when present during the initial 24 hours of incubation. This work suggested that in man IL-1 plays a necessary role during initial B cell activation without which the development of responsiveness to T cell factors does not occur. Although the exact relationship between IL-1 and BCGF in B cell activation has not been delineated, the data indicate both cytokines exert an effect before initial cell division.

Recently, the data of Oliver *et al.* (1985) and Rabin *et al.* (1985) have questioned the role of BCGF. Both groups of investigators have suggested that BCGF, recently renamed B cell stimulatory factor-1 (BSF-1) (Paul, 1983), may not function primarily as a growth factor but instead may prepare B cells to respond to anti-Ig-mediated activation. This is based on the findings that BSF-1 directly induces B cells to increase expression of class II MHC antigens and perhaps size (Noelle *et al.*, 1984; Roehm *et al.*, 1984). These data have been interpreted as suggesting that the role of BSF-1 is to prepare the B cell to receive an antigenic stimulus (Oliver *et al.*, 1985). The role of BSF-1 following the delivery of the initial preparative signal remains to be precisely determined. However, a number of other activities attributed to BSF-1 have been described. These include costimulation of proliferation with anti-IgM antibodies (Howard *et al.*, 1982), induction of the expression and secretion of IgG1 and IgE in the presence of LPS (Vitetta *et al.*, 1985; Lee *et al.*, 1986), and finally, stimulation of the proliferation of certain T cells and mast cells (Lee *et al.*, 1986). It should be noted that a human lymphokine with amino acid homology to murine BSF-1 has recently been described (Yokota *et al.*, 1986). However, a factor capable of inducing class II MHC antigen expression in the absence of additional accompanying activation events has not yet been reported (Kehrl *et al.*, 1985; Bowen *et al.*, 1986).

The sequence of events that occurs during anti-Ig-stimulated human B cell activation appears to be similar to that described in the murine system by Howard and Paul (1983). Thus, Maraguchi *et al.* (1984b) found that stimulation of tonsillar B cells with suboptimal concentrations of $F(ab')_2$ anti-μ was sufficient to allow cell enlargement and RNA synthesis, but entry into the S phase was dependent upon BCGF. SA has also been utilized to examine the effect of T cell-derived lymphokines on human B cell activation. Whereas SA induces initial B cell DNA synthesis in a T cell-independent manner, Muraguchi and Fauci (1982) reported that supernatants of mitogen-stimulated human peripheral blood mononuclear cells (PBM) augmented the proliferation of SA-activated B cells in a manner analogous to the

BCGF-mediated augmentation of anti-Ig-stimulated B cell proliferation.

Following the development of the B cell costimulator assay in the murine system by Howard *et al.* (1982), a similar assay was developed in the human system by Muraguchi and Fauci (1982). A two-stage culture system was employed. This involved an initial activation of peripheral blood B cells with anti-Ig or SA followed by assay of the ability of T cell supernatants to drive proliferation of the activated cells. Consequently these studies analyzed the effect of T cell-derived lymphokines on the responsiveness of activated human B cells. The role of T cell-derived lymphokines in the initial activation of human B cells has also been examined (Jelinek and Lipsky, 1985). Cell cycle analysis following staining with acridine orange was utilized to examine the role of T cell factors in the initial activation of human peripheral blood B cells stimulated by SA. In these experiments (Table I), a similar percentage of cells stimulated with SA alone or SA in the presence of T cell lymphokines entered G_1 during the first 24 to 36 hours of culture. Similar results were seen when B cell activation was analyzed by acquisition of a number of surface markers of cell-activation such as expression of transferrin receptors (Jelinek and Lipsky, 1985) which are not expressed on resting B cells but rather are acquired during the G_1 phase of the cell cycle (Kehrl *et al.*, 1984; Haynes *et al.*, 1981). The data obtained using SA as the PBA are therefore consistent with the results obtained using anti-Ig as a stimulus in that B cells can proceed into the G_1 phase of the cell cycle in the absence of T cell influences.

Entry into the S phase by anti-μ-stimulated tonsillar B cells has been claimed to require BCGF (Muraguchi *et al.*, 1984b). By contrast,

TABLE I
B CELL ENTRY INTO THE CELL CYCLE: THE ROLE OF T CELL FACTORS[a]

	Percentage of cells in the G_1 phase			
	Addition to culture			
Length of incubation	Nil	T cell supernatant	SA	SA + T cell supernatant
24 hr	2.1 ± 0.9	8.0 ± 3.3	12.8 ± 2.8	12.3 ± 2.8
36 hr	5.6 ± 1.2	15.5 ± 2.4	38.3 ± 2.7	38.5 ± 2.3

[a] B cells were cultured with various stimuli for 24 or 36 hr, stained with acridine orange, and analyzed for RNA content. Data represent mean ± SEM of four experiments. (From Jelinek and Lipsky, 1985.)

SA-activated peripheral blood B cells were able to proceed into the S phase without T cell influences (Fig. 5). T cell lymphokines were found to enhance progression of some B cells through the first S phase, but the major effect of T cell lymphokines became apparent after the first round of cell division, when these factors became absolutely necessary to maintain the growth of the activated B cells. Whereas T cell factors appeared to play a minimal role in increasing the total number of SA-stimulated B cells entering into or progressing through the initial cell cycle, it is possible that Mϕ or T cell-derived factors play a more important role during the first cell cycle when other stimuli are used to activate B cells (Muraguchi *et al.*, 1984b). The data, however, support the conclusion that when SA is the polyclonal B cell activator, T cell factors appear to play a minimal role in increasing the total number of SA-stimulated B cells entering into or progressing through the initial cell cycle, thus suggesting that there is no obligatory need for T cell lymphokines during initial B cell activation. These lymphokines, however, may facilitate responsiveness when activation signals are suboptimal.

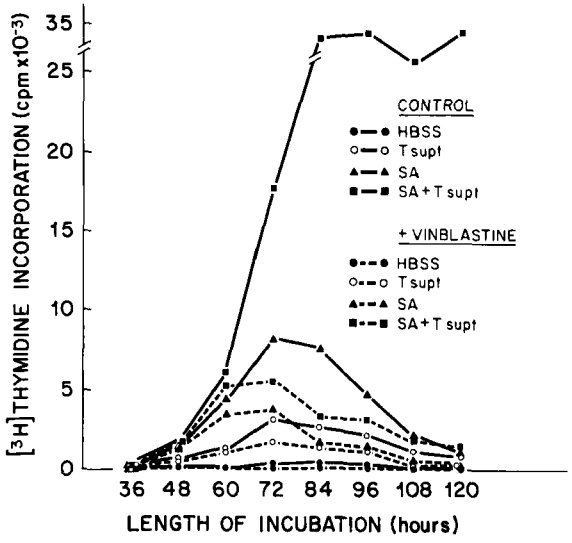

FIG. 5. The role of T cell factors in promoting continued DNA synthesis by SA activated B cells. B cells were cultured with Hanks' balanced salt solution (HBSS), mitogen activated T cell supernatant (T supt), SA, or SA + T cell supernatant in the presence (dashed lines) or absence (solid lines) of vinblastine (added at 24 hr). [^{3}H]Thymidine incorporation was determined after 36–120 hr of culture. From Jelinek and Lipsky (1985).

IV. Responsiveness of B Cell Subpopulations

A number of characteristics have been employed as a means to define functional B cell subpopulations. One method is predicated on the observation that B cell subsets manifest differential expression of a variety of cell surface antigens (reviewed by McKenzie and Zola, 1983). One of the earliest systems in which B cell heterogeneity was studied was that of the CBA/N mouse strain, which possesses a mutant X-linked gene (*xid*) that results in a set of deficiencies in B cell responsiveness. The inability of *xid* mice to respond to a class of T cell-independent antigens (TI-2 antigens) as well as anti-Ig antibodies has been shown to result from the absence of the $Lyb5^+$ subset of B cells (Huber *et al.*, 1977). This population of cells is a more mature subset of B cells than $Lyb5^-$ B cells. Although it has been suggested that only the $Lyb5^-$ B cell subset required MHC-restricted T cell help and that the $Lyb5^+$ B cells may respond directly to T cell factors (reviewed by Singer and Hodes, 1983), this finding has recently been challenged by Mosier and Feeney (1984) and Asano *et al.* (1985).

An Lyb5 equivalent has not yet been described in man. A number of other B cell-specific antigens have been utilized, however, in attempts to study human B cell heterogeneity. Among the first of these markers to be described were the B1 and B2 antigens (Stashenko *et al.*, 1980; Nadler *et al.*, 1981b). Although the vast majority of resting B cells express both B1 and B2, irrespective of anatomic origin of the B cells, some differences do exist in the expression patterns of these two antigens. The B1 antigen is first detected on the surface of pre-B cells before the development of cytoplasmic μ chains (Hokland *et al.*, 1983; Nadler *et al.*, 1984) and is lost at the secretory stage of B cell differentiation (6–7 days following stimulation with PWM; Stashenko *et al.*, 1981). The B2 antigen is lost somewhat earlier than B1 following activation (4–5 days after PWM stimulation) and correlates with loss of surface IgD expression (Stashenko *et al.*, 1981). In addition, B2 is acquired on pre-B cells following the development of cytoplasmic μ chains (Nadler *et al.*, 1984). The B2 antigen has recently been shown to bind the C3d component of complement (Iida *et al.*, 1983) and is therefore closely associated with the CR2 receptor, which is thought to function as the receptor for Epstein–Barr virus (Fingeroth *et al.*, 1984; Nemerow *et al.*, 1985b).

Recent work by Nemerow *et al.* (1985a) has shown that some monoclonal anti-CR2 antibodies directly stimulated peripheral blood B cell proliferation and differentiation in a T cell-dependent manner. Monoclonal antibodies to B1 have also been shown to stimulate tonsillar B

cell proliferation, thus suggesting a role for the B1 antigen in activation (Clark *et al.*, 1985). The exact role of B1 in B cell activation remains controversial, however, in that similar studies by Tedder and co-workers (1985) demonstrated that anti-B1 monoclonal antibodies by themselves could not directly stimulate B cells and were inhibitory when added into anti-Ig-, SA-, or EBV-activated B cells.

In addition to monitoring expression of these two antigens as a means of analyzing B cell activation and defining distinct stages of B cell differentiation (Stashenko *et al.*, 1981), expression of these two antigens has provided a means to analyze heterogeneity of responsiveness. For example, human splenic $B1^+$ $B2^+$ B cells were found by Anderson and colleagues (1985) to respond to anti-Ig or anti-Ig and T cell factors but not to T cell factors alone. By contrast, the $B1^+$ $B2^-$ cells did not proliferate to anti-Ig with or without T cell factors but did secrete Ig upon coculture with $CD4^+$ T cells in the absence of mitogen, suggesting that these cells may be a more differentiated subpopulation.

A number of other B cell surface antigens have been identified and was the subject of a recent review by McKenzie and Zola (1983). Spontaneous rosette formation with mouse erythrocytes (Stathopoulos and Elliott, 1974) has been useful in delineating a subset of human B lymphocytes (Siegel *et al.*, 1971). Further examination by Lucivero and colleagues (1981) indicated that the mouse erythrocyte rosette-positive subpopulation of peripheral blood B cells expressed surface IgM, IgD, and C3 receptors and responded poorly to PWM. Additionally, the rosette-positive cells were found to contain low percentages of $sIgG^+$ and $sIgA^+$ cells whereas the rosette-negative subset was enriched for $sIgG^+$ and $sIgA^+$ cells, suggesting that they were a population of less mature B cells. Further segregation of responsiveness on the basis of rosette formation was revealed by the work of Ito and Lawton (1984), who found that the proliferative response to SA was almost entirely restricted to the rosette-positive population whereas Ig production stimulated by PWM or SA in the presence of T cells was much greater by the rosette-negative B cells.

Differential activation requirements have been observed for B cells separated into subsets on the basis of size (Shortman *et al.*, 1976; DeFranco *et al.*, 1982b; Gorczynski and Feldman, 1975; Watkins *et al.*, 1985; Press *et al.*, 1977; Thompson *et al.*, 1984). In general, these studies have suggested that the small cell population represents a less mature resting B cell population that exhibits more stringent activation requirements than the large, presumably activated B cells. In addition, B cell subsets have also been identified by their differential

ability to respond to various antigens, mitogens, and T cell-derived helper factors (Scher, 1982; Gronowicz and Coutinho, 1976; Swain *et al.*, 1983), but the phenotype of the responding cells has not always been completely defined.

There is a small population of B cells in man that can respond directly to T cell factors in the absence of additional stimulation (Principato *et al.*, 1983; Kuritani and Cooper, 1983; Peters and Fauci, 1983; Chiorazzi *et al.*, 1979; Jelinek and Lipsky, 1985; Bich-Thuy and Fauci, 1985; Bich-Thuy *et al.*, 1985; Jelinek *et al.*, 1986a) although the majority of cells require both T cell factors and a polyclonal B cell activator. The subpopulation of T cell factor-responsive B cells appears to represent B cells that have already undergone an activation step *in vivo* (Principato *et al.*, 1983; Kuritani and Cooper, 1983; Peters *et al.*, 1985), as suggested by the finding that they are larger in size and of lower density (Principato *et al.*, 1983; Kuritani and Cooper, 1983; Peters *et al.*, 1985). Kuritani and Cooper (1983) suggested that the T cell factor-responsive B cells lacked expression of cell surface IgD since anti-IgD antibody did not block this response. The concept that only large activated B cells respond to T cell factors was challenged by the finding that removal of B cells expressing activation antigens from peripheral blood B cells did not eliminate the response to T cell factors alone (Jelinek *et al.*, 1986a). Similarly, in the murine system, the B cell responsive to BSF-1 is thought to be small and resting (Oliver *et al.*, 1985; Rabin *et al.*, 1985), thus additionally challenging the notion that only activated B cells are responsive to T cell factors.

Responsive B cell subsets have most commonly been defined by the expression of cell surface Ig isotype (McKearn *et al.*, 1982; Vitetta *et al.*, 1980, 1984b; Teale *et al.*, 1981). Separation of B cells on the basis of cell surface Ig isotype has been useful in studying the relation of the isotype of surface and secreted Ig. It has generally been found that IgM-bearing B cells give rise to cells that are capable of secreting all isotypes of Ig, whereas cells expressing other isotypes, such as IgG in addition to, or in place of IgM, are more specialized for production of the non-IgM cell surface isotype (reviewed by Teale and Klinman, 1984).

The expression of cell surface IgD during various stages of B cell maturation, activation, and differentiation has been extensively examined. Neonatal B cells have been shown to lack IgD (Abney *et al.*, 1978), whereas expression of surface IgD has often been suggested to correlate with increased resistance to tolerance induction (Kettman *et al.*, 1979; Dosch *et al.*, 1979). In addition, activated B cells and postswitch memory cells both exhibit decreased expression of cell

surface IgD (Coffman and Cohn, 1977; Black *et al.*, 1978, 1980; Preud'homme, 1977; Monroe *et al.*, 1983). Because of the variability of surface IgD expression with maturation and activation, a number of studies have sought to determine whether there is a correlation of B cell surface IgD expression with responsiveness to polyclonal B cell activators. In the murine system, it has been shown that IgM^+, IgD^+ B cells are the primary cells that can be stimulated by lipopolysaccharide (Gronowicz *et al.*, 1979). In man, PWM has been extensively utilized to study response patterns of B cell subsets defined on the basis of cell surface IgD expression. Kuritani and Cooper (1982) as well as others (Saiki and Ralph, 1982; Lucivero *et al.*, 1981) have suggested that PWM-responsive B cells have little or no surface IgD. This conclusion remains controversial as data have also been presented to suggest that PWM may stimulate Ig secretion from IgD^+ human splenic B cells (Finkelman and Lipsky, 1978).

The PWM-responsive subset of human peripheral blood B cells, in addition to being IgD^-, has been described as being IgG^+, Leu 8^-, $4F2^+$, large in size, and of low density (Kuritani and Cooper, 1982; Ault and Towle, 1981; Dagg and Levitt, 1981; Kansas *et al.*, 1985), all of which are characteristics of cells that may have been activated *in vivo* (Kuritani and Cooper, 1982; Dagg and Levitt, 1981; Kansas *et al.*, 1985). It is possible that a population of postswitch, IgD^- memory cells rather than recently activated virgin B cells also contributes to these responses. This contention is supported by the observation that PWM stimulates the production of IgA and IgG early in culture and often in parallel with IgM secretion, with no evidence of mitogen-induced isotype switching (Lipsky, 1980; Ginsberg *et al.*, 1978; Stevens *et al.*, 1981). This finding suggests that at least some of the PWM-responsive cells may be postswitch memory cells.

The surface IgD phenotype of the B cell responding to SA has not been studied in detail. However, the possibility has been raised that IgD^+ and IgD^- B cells may both be triggered by SA (Saiki and Ralph, 1982; Mudde *et al.*, 1984; Harada *et al.*, 1982) although responsiveness of cells obtained from different anatomic sites may vary. Thus, tonsillar $sIgD^+$ and $sIgD^-$ B cells manifested comparable proliferative responses to SA (Harada *et al.*, 1982), whereas the proliferative response of peripheral blood B cells to SA has been reported to be largely contained within the $sIgD^+$ subset (Saiki and Ralph, 1982). The surface IgD phenotype of B cells that differentiate into ISC after stimulation by SA in the presence of T cell help has also not been convincingly demonstrated.

Few studies have directly compared the responses of isolated IgD^+

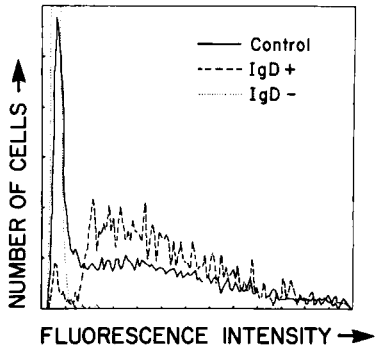

FIG. 6. Separation of B cells into IgD^+ and IgD^- subpopulations. Highly purified B cells were analyzed on the fluorescence activated cell sorter following staining with monoclonal anti-IgD antibody. The fluorescence profiles of the IgD^+ and IgD^- subsets following separation on the fluorescence activated cell sorter were also obtained. From Jelinek *et al.* (1986a).

and IgD^- B cells to PWM or SA and T cell factors. In an attempt to investigate in more detail the phenotypic characteristics of human peripheral blood B cells induced to secrete Ig by various stimuli, the fluorescence-activated cell sorter was used to separate highly purified B cells (>90% B1 positive, Jelinek and Lipsky, 1985) into IgD^+ and IgD^- subsets (Fig. 6). Analysis of the responsiveness of IgD^+ and IgD^- B cells indicated that both subsets responded to SA + T cell factors and differentiated into approximately equal numbers of Ig-secreting cells (Table II). Both subsets also synthesized DNA in response to SA or SA + T cell factors in an approximately equivalent fashion. Small numbers of Ig-secreting cells were generated in unseparated B cell cultures in response to T cell factors in the absence of SA. Of interest, only the IgD^- subset generated substantial numbers of Ig-secreting cells in response to T cell factors alone (Table II). When the protocol used to obtain IgD^- B cells was altered to ensure that the IgD^- subset contained only IgD^- B cells and not IgD^- contaminating cells such as T cells or Mϕ, it was again found that B cells responsive to SA and T cell factors were contained within the IgD^+ and IgD^- cell subsets. T cell factor-responsive ISC precursors resided only within the IgD^- subpopulation and their responsiveness was not dependent on the presence of contaminating non-B cells. The direct comparison between the responsiveness of IgD^+ and IgD^- B cells (Jelinek *et al.*, 1986a) strongly supports the conclusion that both B cell subsets can be stimulated by SA and T cell factors to undergo DNA synthesis and generate Ig-secreting cells.

TABLE II
RESPONSIVENESS OF IgD^+ AND IgD^- B CELLS TO T CELL SUPERNATANT ALONE AND SA + T CELL SUPERNATANT[a]

Assay population	Addition to culture			
	Nil	T supt	SA	SA + T supt
	(ISC per 10^6 B cells × 10^{-3})			
ISC generation				
Control	0	7.8	0.2	111.2
Stained	0	4.2	0	114.4
IgD^+ (96.0)	0	0.6	0.4	172.0
IgD^- (1.0)	0	24.8	0.4	126.4
	[^{3}H]Thymidine incorporation (cpm × 10^{-3})			
DNA synthesis				
Control	0.2	1.5	3.2	36.3
Stained	0.2	1.6	4.5	28.3
IgD^+ (96.0)	0.2	1.0	5.1	32.0
IgD^- (1.0)	0.4	3.5	3.8	32.2

[a] Control (unsorted) B cells, B cells reacted with anti-IgD but not sorted, and B cells sorted into fluorescence-positive and -negative subsets after reacting with monoclonal anti-IgD antibody were cultured alone or with SA in the presence or absence of mitogen-activated T cell supernatant (T supt). Numbers in parentheses indicate the percentage of fluorescence-positive cells found in the IgD^+ and IgD^- populations upon postsort analysis. [^{3}H]Thymidine incorporation and the number of the Ig-secreting cells (ISC) generated were assayed on day 5. Adapted from Jelinek *et al.* (1986a).

It has been shown that stimulation of peripheral blood B cells with SA + T cell help results in the production of IgM, IgG, and IgA (Harada *et al.*, 1982; Jelinek *et al.*, 1986a). Separation of B cells into IgD^+ and IgD^- B cell subsets revealed differences in the isotypes of Ig secreted by the IgD^+ and IgD^- B cell subsets (Table III). Thus, the stimulated IgD^+ subset secreted predominately IgM, whereas the IgD^- subset was found to be enriched for precursors of IgA- and IgG-producing cells. The finding that IgD^+ B cells secreted predominantly IgM in response to SA + T supt is consistent with the work of Saiki and Ralph (1982), who demonstrated that depletion of $sIgD^+$ cells from an unseparated mononuclear cell population resulted in a de-

creased number of IgM-secreting cells in response to stimulation with SA alone or SA + PWM with little effect on the number of IgA- or IgG-secreting cells generated. By contrast tonsillar IgD^+ B cells could not be induced to secrete Ig of any class following stimulation with SA alone, although optimal T cell help may not have been available (Harada *et al.*, 1982). Analysis of responsiveness of human IgD^+ and IgD^- B cell subsets has suggested that IgD^- B cells manifest less stringent activation requirements compared to IgD^+ B cells (Kuritani and Cooper, 1982; Lucivero *et al.*, 1981). It is important to point out that these observations have been made using PWM as the stimulus. Investigators utilizing SA as the stimulus have not demonstrated preferential stimulation of IgD^- B cells (Jelinek *et al.*, 1986a). The capacity of SA to stimulate IgD^+ B cells may result in part from the powerful surface Ig cross-linking effect of SA (Romagnani *et al.*, 1981; Forsgren *et al.*, 1976).

As mentioned above, it has previously been suggested that PWM-responsive B cells have little or no surface IgD (Kuritani and Cooper, 1982; Saiki and Ralph, 1982; Lucivero *et al.*, 1981). The methods employed in these studies included depletion of IgD^+ B cells from whole mononuclear cell preparations by panning or selection on the

TABLE III
Ig ISOTYPE SECRETED BY B CELL SUBSETS STIMULATED WITH SA + T CELL SUPERNATANTS[a]

Experiment	Population	Secreted immunoglobulin (ng/ml)		
		IgM	IgG	IgA
1	Control	637	430	144
	IgD^+	749	49	23
	IgD^-	20	669	170
2	Control	1280	220	88
	IgD^+	940	0	0
	IgD^-	240	472	680

[a] Control unsorted B cells and B cells sorted into fluorescence-positive and -negative subsets after reacting with anti-IgD were cultured for 5 days with SA + T cell supernatant before carrying out ELISA determinations of secreted Ig. Unstimulated cells contained undetectable levels of all three isotypes of Ig. Adapted from Jelinek *et al.* (1986a).

TABLE IV
IgD$^+$ AND IgD$^-$ SUBSET RESPONSIVENESS TO PWM: ROLE OF T CELLS AND MONOCYTES[a]

		ISC per 10^6 B cells ($\times 10^{-3}$)	
		Addition to culture	
Population	Addition	HBSS	PWM
Control	—	0	0
	Mϕ	0.5	0.5
	T mito	0	80.0
	Mϕ + T mito	7.5	233.5
Stained	—	0	0
	Mϕ	0	0.5
	T mito	0	92.5
	Mϕ + T mito	2.0	238.0
IgD$^+$	—	0.5	0
	Mϕ	0	0
	T mito	0	28.5
	Mϕ + T mito	0	69.0
IgD$^-$	—	0	0
	Mϕ	0.5	2.5
	T mito	5.0	406.0
	Mϕ + T mito	6.5	892.0

[a] Control unsorted and stained nonsorted B cells and B cells sorted into fluorescence-positive and -negative subsets after reacting with anti-IgD were cultured (1×10^4/well) in the presence or absence of PWM; 5000 irradiated autologous Mϕ or 50,000 mitomycin C-treated T cells (T mito) were added to each well where indicated. Adapted from Jelinek *et al.* (1986a).

fluorescence-activated cell sorter, depletion using anti-Ig-coated bovine RBC, and separation of B cells into mouse RBC rosette-positive and rosette-negative cells. Direct functional comparisons between IgD$^+$ and IgD$^-$ peripheral blood B cell subsets were performed by Jelinek *et al.* (1986a) using the fluorescence-activated cell sorter to select IgD$^+$ and IgD$^-$ B cells from an initial highly purified B cell preparation. The results shown in Table IV confirm the T cell dependence of the PWM response (Keightley *et al.*, 1976) and that Mϕ amplify PWM responsiveness (Rosenberg and Lipsky, 1979). Consistent with reports in the literature, the IgD$^-$ subset was greatly en-

TABLE V
Ig ISOTYPE SECRETED BY PWM-STIMULATED IgD$^+$ AND IgD$^-$ B CELLS[a]

Experiment	Population	PWM-stimulated Ig secretion (ng Ig secreted/ml) IgM	IgG	IgA
1	Control	120	253	147
	IgD$^+$	205	0	0
	IgD$^-$	533	1333	1333
2	Control	680	248	1640
	IgD$^+$	320	0	31
	IgD$^-$	680	1040	1700

[a] Control unsorted B cells and B cells sorted into fluorescence-positive and -negative subsets after reacting with anti-IgD were cultured at 1×10^4/well with PWM and 50,000 mitomycin C-treated T cells/well and 5000 irradiated Mϕ/well. ELISA determinations of secreted Ig were performed on cell-free culture supernatants collected after a 5-day incubation. Adapted from Jelinek *et al.* (1986a).

riched for PWM-responsive precursors of Ig-secreting cells. Comparison of IgD$^-$ B cells with control unseparated B cells demonstrated a 2- to 17-fold enhancement in the number of Ig-secreting cells generated in response to PWM (Jelinek *et al.*, 1986a; Table IV). Although the IgD$^+$ B cell subset responded poorly to PWM and the number of Ig-secreting cells generated was small in comparison to the number found in either control or IgD$^-$ B cell cultures, responsiveness of the IgD$^+$ B cell subpopulation to PWM was consistently observed. When the isotype of secreted Ig was analyzed (Table V), PWM-responsive ISC precursors present in the IgD$^+$ subset were limited to IgM secretion. The IgD$^-$ B cells secreted large amounts of IgA and IgG, but IgM secretion was also stimulated from this subset. In fact, more IgM was secreted by the IgD$^-$ than the IgD$^+$ B cell subset. Experiments carried out by Kuritani and Cooper (1982) have suggested that anti-IgD antibodies may actually enhance PWM-induced differentiation. In the experiments described in Table IV, it was unlikely that the anti-IgD antibodies were stimulatory since anti-IgD stained but unsorted B cells manifested responses that were comparable to those of control B cells.

Work by Kuritani and Cooper (1982, 1983) and Principato *et al.* (1983) has demonstrated that B cells responding directly to T cell factors or PWM are predominantly low density cells, suggesting that they may have undergone an initial activation step *in vivo*. These studies do not directly distinguish between the possibility that the cells stimulated by PWM or T supt were large, activated B cells or alternatively whether the responsive cells were IgD^- postswitch precursors of IgA and IgG secretion. The finding that isolated IgD^- peripheral blood B cells are enriched in cells expressing cell surface IgG and IgA (Jelinek *et al.*, 1986a) supports this latter possibility. To evaluate this, cell volume and the activation marker status of IgD^+ and IgD^- B cells were analyzed. The mean volume of the IgD^- B cells was found to be somewhat greater than the mean volume of the IgD^+ cells (203 μm^3 vs 177 μm^3), indicating that the IgD^- cells contained larger cells (Jelinek *et al.*, 1986a). Additional characterization indicated that approximately 12% of freshly isolated IgD^- cells exhibited an RNA content commensurate with early G_1 whereas the IgD^+ population was uniformly composed of cells in G_0. In addition, the expression of two markers which have been reported to be absent on resting B cells but expressed in early G_1 (4F2) and late G_1 (5E9) (Kehrl *et al.*, 1984) were also examined. Although there was no evidence of 5E9 expression in any population, the IgD^- subset was shown to contain about 10–15% 4F2-positive cells, whereas IgD^+ cells were found to be devoid of reactivity with 4F2. Thus, although the IgD^- subset was largely resting, a small population of activated cells was also found. These findings are consistent with the work of others (Kuritani and Cooper, 1982; Lucivero *et al.*, 1981; Dagg and Levitt, 1981). Recent work by Kansas *et al.* (1985) indicated that the PWM-responsive B cell was not identified by the Leu8 monoclonal antibody and that an average of 39% of $Leu8^-$ B cells also expressed 4F2. Although an association between PWM responsiveness and 4F2 expression was suggested by this report, PWM responsiveness of the $4F2^+$ subpopulation was not examined. Since the precursor frequency of the PWM-responsive subpopulation is small (Martinez-Maza and Britton, 1983), it is possible that such precursors might not be found uniquely in the $4F2^+$ population. Indeed, work by Jelinek *et al.* (1986a) demonstrated that responsiveness to T cell factors, SA + T cell factors, and PWM was still observed in the IgD^- subset even following removal of the $4F2^+$ B cells (IgD^- $4F2^-$ subset). Data provided in this study indicate resting as well as activated IgD^- B cells respond to T cell factors, PWM, and SA + T cell factors.

These studies have provided information regarding the response characteristics of resting virgin B cells and those of circulating memory cells. Findings obtained from these studies (summarized in Fig. 7) indicate that the IgD^+ population was largely limited to IgM secretion regardless of the nature of the stimulus, suggesting that these cells may represent a less experienced population of circulating virgin B cells. These cells have not yet reached a sufficient state of maturation that allows them to respond directly to T cell factors. Moreover the majority of them are unable to respond to PWM. The demonstration that the IgD^- B cells were enriched in cells responding to T cell factors alone or PWM in the presence of T cells and the finding that they were also enriched for postswitch precursors of IgG- and IgA-secreting cells indicate that they were a more mature subset of B cells. Heterogeneity in the degree of activation could be found in the IgD^- population. Thus, both a resting $4F2^-$ and an activated $4F2^+$ B cell were found in the IgD^- subset. Removal of the activated $4F2^+$ B cells still permitted IgM, IgG, and IgA secretion, supporting the conclusion that the IgD^- population contained a subset of resting memory B cells.

It remains a matter of controversy whether PWM-induced B cell responses require a direct interaction of B cells with T cells or whether T cell-derived soluble factors can substitute for intact T cells

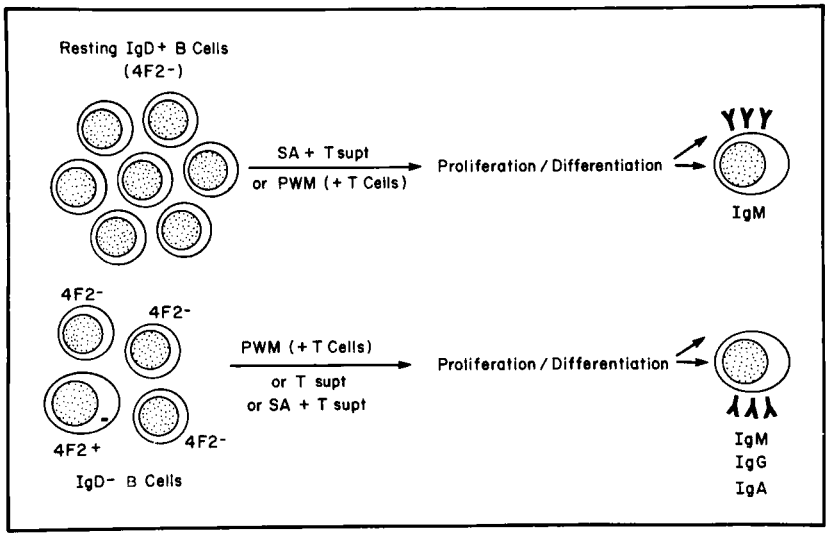

FIG. 7. Schematic representation of the responsiveness of IgD^+ and IgD^- cell subsets.

(Fauci *et al.*, 1976, 1980; Lipsky, 1985; Suzuki *et al.*, 1986; Cueppens and Stevens, 1986). It has been suggested that PWM-induced B cell responses are primarily driven by soluble factors produced by the T cells present in the PWM-stimulated cultures (Kuritani and Cooper, 1982; Hirano *et al.*, 1977). It appears likely, however, that PWM plays an additional role in B cell responses besides inducing the production of necessary T cell factors. Although some investigators have shown that soluble T cell factors can support a PWM-driven response (Fauci *et al.*, 1980; Cueppens and Stevens, 1986), experimental systems that have employed more highly T cell- and monocyte-depleted B cells have not been able to demonstrate reproducibly that the requirement for T cells could be replaced by soluble factors (Suzuki *et al.*, 1986).

The issue of whether the same population of B cells responded to both soluble T cell factors alone and PWM in the presence of T cells was analyzed by comparing the magnitude of responses obtained in IgD^- cell cultures stimulated by T cell factors in the presence or absence of supplemental T cells or with PWM in the presence of T cells (Jelinek *et al.*, 1986a). It was shown (Table VI) that PWM in the

TABLE VI
EFFECT OF T CELLS ON B CELL DIFFERENTIATION INTO ISC[a]

			ISC per 10^6 B cells ($\times 10^{-3}$)	
			Addition to culture	
Experiment	Population	Addition	T supt	PWM
1	Control	—	1.8	ND
		T mito	32.8	24.5
	IgD^-	—	0.5	ND
		T mito	36.5	192.0
2	Control	—	12.2	ND
		T mito	64.0	88.5
	IgD^-	—	24.4	ND
		T mito	65.0	743.0

[a] Control unsorted B cells and fluorescence-negative B cells selected on the FACS after reacting with anti-IgD, OKT3, OKT11, and 63D3 were cultured at 2.5×10^4/well when stimulated with mitogen-activated T cell supernatant (T supt) and at 1×10^4/well when stimulated with PWM in the presence or absence of 50,000 mitomycin C-treated T cells per well (T mito). The number of ISC generated was assayed on day 5. ND, Not determined. From Jelinek *et al.* (1986a).

presence of T cells but not T cell factors induced 5- to 10-fold greater responses than T cell factors even in the presence of intact T cells. These results support the conclusion that a direct PWM-mediated T cell–B cell interaction is required in these responses. PWM may serve to facilitate T–B interactions in a manner analogous to the linked recognition necessary to activate hapten-specific murine B cells (Noelle *et al.*, 1983) although such an interaction is antigen nonspecific and MHC unrestricted. Recent work by Suzuki and colleagues (1986) has shown that the PWM response of human peripheral blood B cells requires at least an initial 6-hour interaction between B and T cells in the presence of monocytes and that this interaction is blocked by the addition of anti-Ia or anti-CD-4 antibodies. Following this initial activation, the B cells could then be driven by soluble factors. Our own preliminary findings support the conclusion that recognition of nonpolymorphic Ia determinants is involved in this interaction and also suggest a role for lymphocyte function-associated (LFA-1) molecules (unpublished observations).

V. Factors Affecting B Cell Activation, Proliferation, and Differentiation

Investigation of the role of cytokines in B cell activation, proliferation, and differentiation has led to the description of a number of biologically active factors. T cell-derived B cell growth and differentiation factors have been the topic of a number of recent review articles (Muraguchi *et al.*, 1984a; Howard *et al.*, 1984; Mayer *et al.*, 1984; Kishimoto *et al.*, 1984; Vitetta *et al.*, 1984a; Kishimoto, 1985).

One of the first lymphokines to be described with an apparent exclusive action on B cells was B cell growth factor. BCGF was classically defined in the murine system as the T cell-derived soluble factor that synergized with anti-Ig antibodies to induce B cell proliferation (Howard *et al.*, 1982). This activity was separated from other lymphokines and determined to have a molecular weight of 17,000–20,000 (reviewed by Howard and Paul, 1983; Kishimoto, 1985). The gene encoding BCGF has recently been cloned (Noma *et al.*, 1986; Lee *et al.*, 1986) and the product of this gene has been reported to have a molecular weight of 20,000. This gene product exerts a number of biologic activities, including induction of class II MHC antigens on resting B cells, costimulation with IgM antibodies, enhancement of the production of IgG_1 and IgE in LPS-stimulated cultures, and stimulation of some T cell and mast cell lines (Lee *et al.*, 1986; Noma *et al.*, 1986). It has not yet been determined whether this molecule exerts its several effects on B cells at a single point in the activation scheme or

at several or whether different subpopulations of B cells are stimulated by BSF-1.

Following the description of a second higher molecular weight (M_r 50,000–70,000) BCGF (Okada *et al.*, 1983; Swain *et al.*, 1983), the low-molecular-weight substance was designated BCGF I (BSF-1) and the designation of BCGF II was assigned to the high-molecular-weight form. The discrimination between the two BCGF molecules was made on the basis of molecular weight and on functional characteristics. Murine B cells stimulated with anti-IgM were augmented in growth by BCGF I (BSF-1) but not by BCGF II. Conversely, B cells stimulated with dextran sulfate proliferated in response to BCGF II but not BCGF I (Swain *et al.*, 1983). Recent work by Sanderson *et al.* (1986) has suggested that eosinophil differentiation factor in the murine system (Sanderson *et al.*, 1985) may be identical to BCGF II.

A similar growth-promoting activity has also been described in the human system using SA- or anti-Ig-stimulated B cells to assay for growth-promoting activities in mitogen-stimulated supernatants of human T cells (Muraguchi and Fauci, 1982; Okada *et al.*, 1983; Yoshizaki *et al.*, 1983). Muraguchi and co-workers (1982) demonstrated BCGF in the M_r 20,000–30,000 fraction and were able to separate this activity from IL-2. It has furthermore been suggested that there are two distinct forms of human BCGF, distinguished on the basis of molecular weight (Yoshizaki *et al.*, 1983; Ambrus and Fauci, 1985). Shimizu *et al.* (1985) have pursued the separation of human BCGFs and have reported an M_r 60,000 fraction containing activity that did not support anti-Ig- or SA-stimulated B cell DNA synthesis but did support dextran sulfate-stimulated murine B cell proliferation and directly supported the growth of large activated human B cells (Shimizu *et al.*, 1985; Kishimoto, 1985). It is important to note that the high-molecular-weight human BCGF also supported differentiation. Therefore, the separation of the two BCGFs into types I and II provide a convenient means to distinguish the high- and low-molecular-weight forms. However, a precise functional discrimination awaits further study. Recently, a human cDNA encoding a 15,000 Da protein with structural and functional homology to murine BSF-1 has been cloned by Yokota and colleagues (1986). The product of this cDNA was found to support the proliferation of human T cells and helper T cell clones as well as the proliferation of anti-IgM-activated human tonsillar B cells and thus is similar to murine BSF-1. However, it has not yet been determined whether the human BSF-1 equivalent has an effect on IgG_1 and IgE production or on class II MHC antigen expression. Of further interest was the finding by these investigators that

proliferation of anti-IgM-stimulated B cells was supported by either cloned BSF-1 or a commercially available preparation of BCGF, whereas SA-stimulated B cell proliferation was supported only by the BCGF preparation. This finding adds support to the suggestion discussed above that there are at least 2 distinct factors in man that support B cell growth.

Evidence that activities present in supernatants of activated T cells were also able to support differentiation of B cells into ISC was first suggested by the early work of Dutton *et al.* (1971) and Schimpl and Wecker (1972). This activity was originally referred to as T cell replacing factor, or TRF. This activity has also been referred to as B cell differentiation factor (BCDF), a term that was originally used to designate those factors that do not support growth but are involved in the final differentiation of activated B cells to high-rate Ig secretion (Isakson *et al.*, 1982). A number of investigators have separated BCDF activity from other activities present in supernatants from mitogen-stimulated T cells or T hybridomas. As with BCGF, human BCDF apparently exists in two molecular weight forms, one of 20,000 and the other of 30,000–35,000 (reviewed by Howard and Paul, 1983; Kishimoto, 1985; Mayer *et al.*, 1983), designated BCDF-I and BCDF-II, respectively. In the human, both species have been found to be required for maximal responsiveness (Hirano *et al.*, 1984). Similar to the multiple activities of murine BCGF-I, some differentiation factors also support B cell growth and hence have been referred to as B cell growth and differentiation factors (BGDF). BGDF activity has been reported in both the human (Shimizu *et al.*, 1985) and murine systems (Pike *et al.*, 1982; Booth *et al.*, 1983; Takatsu *et al.*, 1980).

B cell differentiation factor(s) has been viewed as acting after initial activation and proliferation of stimulated B cells. Thus, BCGF-driven B cell proliferation is thought to generate daughter cells that can differentiate in response to BCDF. This sequence of events remains somewhat speculative, however. Although many studies have confirmed the requirement for BCDF in the generation of Ig-secreting cells from T cell-depleted B cells, the temporal relationship of its activity to other triggering events has been less completely documented. For example, PWM-stimulated B cells have been reported to differentiate into Ig-secreting cells only when a source of BCDF is present from the initiation of culture (Hirano *et al.*, 1977). By contrast, SA-stimulated B cells may not require BCDF until 48–72 hours after initial culture for maximal generation of Ig-secreting cells (Hirano *et al.*, 1984).

The role of differentiation-inducing factors during initial B cell activation was addressed in detail by the studies of Jelinek and Lipsky (1985). A two-stage culture system involving an initial activation of peripheral blood B cells with SA in the presence or absence of T cell factors was utilized in an effort to understand in greater detail the relationship of initial activation and proliferation of B cells to the subsequent differentiation of B cells into Ig-secreting cells. The T cell factors used in these studies contained activities capable of supporting both growth and differentiation of B cells stimulated with SA. To analyze the sequence in which the various signals were delivered, B cells were cultured with medium alone, T cell factors, SA, or SA + T cell factors for 48–72 hours, washed, and recultured for 72 hours in the presence of various additions, after which the generation of Ig-secreting cells was determined. Preincubation with SA followed by culture with T cell factors routinely resulted in the generation of large numbers of Ig-secreting cells (Table VII). The other sequences of stimuli failed to result in the differentiation of Ig-secreting cells, although on occasion preincubation with T cell factors followed by culture with T

TABLE VII
SEQUENCE OF EVENTS IN THE GENERATION OF ISC: SA ACTIVATION PRECEDES THE REQUIREMENT FOR T CELL FACTOR INFLUENCES[a]

Assay	First incubation conditions[b]	Addition during second incubation[c]		
		Nil	SA	T supt
		(ISC per 10^6 B cells × 10^{-3})		
Generation of Ig-secreting cells	0	0	0	0.4
	T supt	0	0	0.6
	SA	0	0	18.8
		[^{3}H]Thymidine incorporation (cpm × 10^{-3})		
B cell DNA synthesis	0	0.1	0.5	0.4
	T supt	0.1	4.6	0.6
	SA	0.4	0.4	15.3

[a] From Jelinek and Lipsky (1985).

[b] B cells were cultured alone, with T cell supernatant (T supt) or SA for varying lengths of time (72, 48, and 48 hr, for expts. 1, 2, and 3, respectively) before washing 3× with HBSS and reculturing with the indicated additions.

[c] Preincubated B cells were cultured alone, with SA, or with fresh T cell supernatant before harvest and assay for the total number of Ig-secreting cells (ISC) and [^{3}H]thymidine incorporation.

cell factors led to differentiation of small numbers of Ig-secreting cells. The finding that B cells incubated first with T cell factors followed by culture with SA did not generate significant numbers of Ig-secreting cells supported the conclusion that SA activation precedes the reception of differentiative signals delivered by T cell factors.

Although T cell factors did not need to be present during the initial B cell activation with SA to permit subsequent differentiation into Ig-secreting cells, it was found that when T cell factors were present during this initial phase, an increased number of Ig-secreting cells was subsequently produced (Table VIII). Results of this nature suggested that although there was no absolute requirement for T cell influences during initial B cell activation, T cell lymphokines can deliver a signal during this period that permits enhanced differentiation of Ig-secreting cells subsequently. In an effort to determine whether the T cell signals that enhanced subsequent B cell differentiation were delivered before or after cell division, experiments utilizing hydroxyurea were undertaken. It was found that T cell factor-mediated augmentation of the generation of Ig-secreting cells in SA-stimulated B cell cultures was observed during the first few hours of B cell activation and was not altered by the presence of HU (Jelinek and Lipsky, 1985). This suggested that the T cell factors committed a portion of the activated B cells before initial entry into the S phase to

TABLE VIII

DIFFERENTIATION OF Ig-SECRETING CELLS FROM SA-ACTIVATED B CELLS: EFFECT OF T CELL FACTORS DURING INITIAL ACTIVATION[a]

Experiment	First incubation[b]		Length of second incubation[c]	ISC per 10^6 B cells ($\times 10^{-3}$)	
				Addition during second incubation	
	Length	Addition		Nil	T supt
1	36 hr	SA	96 hr	0	13.0
		SA + T supt		1.8	61.2
2	48 hr	SA	96 hr	0	18.8
		SA + T supt		0	45.2

[a] From Jelinek and Lipsky (1985).

[b] B cells were cultured with SA or SA + mitogen-activated T cell supernatant (T supt) for varying lengths of time before being washed and recultured in the second incubation.

[c] B cells were cultured in the presence of fresh T cell supernatant for varying lengths of time prior to assay for total number of Ig-secreting cells (ISC).

secrete Ig subsequently. When hydroxyurea was present for the first 48 hours, subsequent generation of Ig-secreting cells was largely eliminated. This is consistent with the findings that generation of Ig-secreting cells requires antecedent DNA synthesis (Jelinek and Lipsky, 1983), although differentiation signals may be conveyed before the initial G_1–S interphase.

Other studies have also suggested that the presence of T cell factors during the period of time before entry into the S phase of the first cell cycle facilitates the subsequent generation of Ig-secreting cells. Thus, for example, Hirano *et al.* (1977) demonstrated that maximal generation of PWM-induced Ig-secreting cells required T cells or T cell products from the initiation of culture. Similarly, in the murine system, Goodman and Weigle (1979) found that maximal LPS-induced generation of Ig-secreting cells developed only when T cells were present during the first 24 hours of culture. On the other hand, a number of studies have reported optimal generation of antibody-forming cells only when the addition of T cell lymphokines was delayed for 24 to 48 hours after the initiation of culture. These observations were made both for antigen-induced murine spleen cell antibody responses (Schimpl *et al.*, 1974; Schimpl and Wecker, 1972; Askonas *et al.*, 1974) and in cultures of human tonsillar (Hirano *et al.*, 1984) or peripheral blood B cells (Lipsky, 1985) stimulated with SA. Similar findings were also obtained in studies employing a human leukemic B cell line (Yoshizaki *et al.*, 1982). The discrepancy between the results reported by the various investigators may relate to the use of partially purified B cells such that there may have been enough T cell contamination to provide an initial signal but not enough to maintain a continued response. Therefore, early consumption of factors produced by residual T cells would result in a requirement for additional T cell factors at 48 hours. The difference may also be explained by the use of B cells obtained from different anatomic sites or, alternatively, to the use of a partially purified B cell differentiation factor preparation. The culture conditions in these experiments may also have been such that the rate of B cell progression through the cell cycle was retarded, resulting in B cells that were still predivisional, even as late as 48 hours of culture.

The data obtained from the HU elimination experiment (Jelinek and Lipsky, 1985) suggest that the transmission of the T cell signal(s) resulting in the augmentation of B cell differentiation to Ig-secreting cells occurs before initial division, most likely during the G_1 phase. This is the phase of the cell cycle when the influence of a number of T cell signals is received by B cells. Thus, for example, Kishimoto *et al.*

(1975) demonstrated that the formation or exposure of acceptor sites on B cells for a differentiation factor occurred in the absence of cell division, and therefore likely in G_1. Moreover, Miki and co-workers (1982) showed that the signal(s) provided by a T cell-replacing factor to synchronized human B lymphoblastoid cells could be transduced only when cells were in the G_1 phase.

The best studied of the Mϕ-derived soluble factors that have been demonstrated to be active in antibody responses is IL-1 (reviewed by Oppenheim and Gery, 1982; Oppenheim *et al.*, 1986). Early studies demonstrated an ability of culture supernatants of human monocytes and activated murine macrophages to enhance the humoral response of T cell-depleted murine spleen cultures to SRBC (Schrader, 1973; Wood and Gaul, 1974; Wood and Cameron, 1975; Koopman *et al.*, 1978). Subsequent studies using anti-IgM have suggested that IL-1 functions as a cofactor with BCGF, allowing the entry of anti-Ig-activated B cells into the S phase (Howard *et al.*, 1983; Hoffmann, 1980b). Work by a number of other investigators has also suggested a role for IL-1 in B cell activation (Corbel and Melchers, 1984; Booth and Watson, 1984; Leibson *et al.*, 1982). It has been suggested that the primary role of IL-1 is the promotion of activation but, alone, it supports neither growth nor differentiation. This latter notion has recently been challenged by Pike and Nossal (1985), who suggested that IL-1 alone can promote both growth and differentiation of single, isolated, hapten-specific B cells.

In man, a role for IL-1 in PWM-stimulated antibody responses has also been most easily demonstrated in cultures of partially T cell-depleted B cells (Rosenberg and Lipsky, 1980; Lipsky *et al.*, 1983) where the addition of Mϕ supernatants was able to augment the number of Ig-secreting cells generated substantially. IL-1 appears to deliver a signal directly to the responding B cell (Lipsky *et al.*, 1983; Lipsky, 1985). Thus, an antibody against human IL-1 was found to inhibit both B cell proliferation and differentiation when present during the first 24 hours of incubation. In similar experiments (Lipsky, 1985), addition of IL-1 to PWM-stimulated cultures augmented responses when added at the initiation of culture but lost the capacity to augment responses when addition was delayed for 24 hours. These data were interpreted as suggesting that IL-1 exerted its major effect on B cells early in culture, before a requirement for helper T cell factors. IL-1 was further postulated, therefore, to play a role in inducing receptors for T cell factors, as had been suggested in the murine system (Hoffmann, 1980b). By contrast to the studies showing that IL-1 played a role in the PWM-stimulated response, an action of

IL-1 in SA-stimulated B cell responses has been more difficult to demonstrate. Thus, Falkoff and colleagues (1983) were unable to show that IL-1 had any effect on SA-stimulated B cell DNA synthesis. When similar studies were carried out using suboptimal concentrations of anti-μ antibody as the stimulus, synergy could be seen between IL-1 and BCGF (Falkoff *et al.*, 1983). In a later study, the same group of investigators showed that IL-1 could enhance the generation of Ig-secreting cells by SA-activated tonsillar B cells cultured with a source of BCDF, but an absolute requirement for IL-1 could not be demonstrated (Falkoff *et al.*, 1984). We have recently found that concentrations of highly purified IL-1 as low as 1 p*M* markedly augmented both proliferation and differentiation of highly purified spleen or peripheral blood B cells stimulated with SA and interleukin 2 (IL-2) although no absolute requirement for IL-1 was observed (Jelinek *et al.*, unpublished observation).

T cell growth factor, or IL-2, has traditionally been viewed as a lymphokine that functions specifically to maintain the growth of activated T cells (Morgan *et al.*, 1976; Smith, 1980). Until recently, a direct role for IL-2 in B cell activation has not been demonstrated (Robb *et al.*, 1981; Kishimoto *et al.*, 1984; Uchiyama *et al.*, 1981). Rather, as discussed above, other T cell-derived lymphokines unrelated to IL-2, such as BCGF and BCDF, have been felt to be involved in the growth and differentiation of activated B cells (Howard *et al.*, 1982; Isakson *et al.*, 1982; Butler *et al.*, 1983; Nakanishi *et al.*, 1983).

Following the demonstration that B cells could be induced to express receptors for IL-2, a number of functional studies suggested that IL-2 might also play a role in B cell activation (Korsmeyer *et al.*, 1983; Tsudo *et al.*, 1984; Zubler *et al.*, 1984; Jung *et al.*, 1984; Waldmann *et al.*, 1984; Nakanishi *et al.*, 1984; Muraguchi *et al.*, 1985; Nakagawa *et al.*, 1985; Lowenthal *et al.*, 1985). This conclusion, however, initially remained controversial with some studies reporting minimal if any obligatory role for IL-2 in B cell responsiveness (Howard and Paul, 1983; Ralph *et al.*, 1984; Pure *et al.*, 1982). Part of the disparate findings could initially be attributed to a lack of sufficiently purified IL-2 for study and the potential contribution of cells contaminating the B cell preparations. The first of these problems has largely been circumvented by the recent availability of IL-2 prepared by recombinant DNA technology (Taniguchi *et al.*, 1983; Wang *et al.*, 1984). Recombinant IL-2 (r-IL-2) has been reported by several investigators to play a role in supporting the growth and differentiation of activated B cells (Zubler *et al.*, 1984; Jung *et al.*, 1984; Waldmann *et al.*, 1984; Nakanishi *et al.*, 1984; Muraguchi *et al.*, 1985; Nakagawa *et al.*, 1985;

Pike *et al.*, 1984; Mingari *et al.*, 1985). In addition, Pike *et al.* (1984) demonstrated that human r-IL-2 was active on single, hapten-specific murine B cells, thus militating against the possibility that the effect of IL-2 on B cells was an indirect one resulting from an action on residual T cells.

Despite the demonstration that IL-2 can promote B cell responsiveness, the magnitude of both the antibody and proliferative responses in cultures supported by IL-2 alone has usually been small in comparison to those maintained by T cell supernatants containing a variety of T cell-derived lymphokines (Jung *et al.*, 1984; Nakagawa *et al.*, 1985; Pike *et al.*, 1984). In addition, the concentrations of IL-2 required to promote B cell responsiveness have usually been large in comparison with those necessary to maintain T cell growth (Jung *et al.*, 1984; Nakanishi *et al.*, 1984; Ralph *et al.*, 1984).

The importance of interferon-gamma (IFN-γ) in supporting B cell proliferation and differentiation into ISC, either in a direct or indirect fashion, also remains unresolved. Several early studies found that IFN-γ suppressed antibody responses (Brodeur and Merigan, 1975; Booth *et al.*, 1976), presumably via an antiproliferative effect. More recent work, however, has indicated that this antiproliferative effect may be related to contaminating lymphotoxin and not IFN-γ itself (Conta *et al.*, 1985). Recently, the availability of recombinant IFN-γ (r-IFN-γ) (Gray *et al.*, 1982) has permitted a reevaluation of the effect of IFN-γ on B cell responsiveness. Mond and colleagues (1985a) showed that r-IFN-γ inhibited the murine B cell proliferative response stimulated by soluble anti-Ig antibody and could also partially suppress the induced expression of Ia. By contrast, a number of studies have suggested that r-IFN-γ may amplify antibody responses in B cell cultures, especially those supported by additional T cell lymphokines (Nakagawa *et al.*, 1985; Nakamura *et al.*, 1984; Leibson *et al.*, 1984; Sidman *et al.*, 1984; Murray *et al.*, 1985). In man, it has been shown that IFN-γ could not support B cell differentiation alone but high concentrations of r-IFN-γ (250–1000 U/ml) could promote antibody secretion in IL-2-supported cultures of mitogen-activated tonsillar B cells (Nakagawa *et al.*, 1985; Bich-Thuy and Fauci, 1986). Results from both of these studies also indicated that in contrast to IL-2, IFN-γ alone did not support B cell growth. In light of these results, it was suggested that IL-2 was a B cell growth factor whereas IFN-γ may be a differentiation factor (Nakagawa *et al.*, 1985). IFN-γ, however, was not active alone in that it could not stimulate Ig production by BCDF-responsive B lymphoblastoid cell lines (Nakagawa *et al.*, 1985).

Considerable controversy remains concerning the activation status of B cells responsive to IFN-γ. For example, Leibson *et al.* (1984) demonstrated that IFN-γ could substitute for a late-acting T cell-derived helper factor which acted synergistically with other helper factors in stimulating a murine B cell response to SRBC *in vitro*. This result suggested that the mode of action of IFN-γ was similar to that of classic T cell-replacing factor (TRF). On the other hand, Brunswick and Lake (1985), also working in the murine system, found that IFN-γ was necessary to allow TRF to exert its effect and that IFN-γ was required during the first 24 hours of incubation for the development of maximal antibody responses to SRBC. Finally, IFN-γ has been claimed to act as a completely sufficient differentiation factor, stimulating the differentiation of Ig-secreting cells from resting murine spleen B cells in the absence of other signals (Sidman *et al.*, 1984).

A schematic depiction of B cell activation based on the work by the aforementioned investigators is shown in Fig. 8. This model incorporates the reported activity(s) of IL-1 as suggested by DeFranco *et al.* (1982b), Howard *et al.* (1983), Hoffmann (1980b), and Lipsky (1985), the activity of BSF-1 (BCGF-I) as suggested by Oliver *et al.* (1985) and Rabin *et al.* (1985), the activity of BCGF-II (Swain *et al.*, 1983), and the proposed function of differentiation promoting factors as suggested by Schimpl and Wecker (1972) and others (reviewed by Howard and Paul, 1983; Kishimoto, 1985). In addition, this model incor-

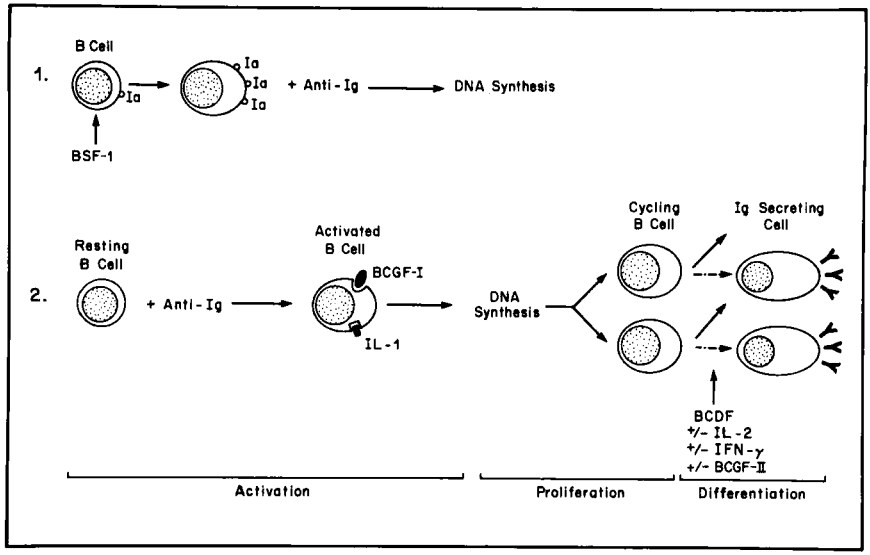

FIG. 8. Consensus view of the role of various lymphokines in B cell activation.

porates the activity of the purified lymphokines, IL-2 and IFN-γ, as has recently been suggested by work of a number of investigators (Tsudo *et al.*, 1984; Zubler *et al.*, 1984; Jung *et al.*, 1984; Waldmann *et al.*, 1984; Nakanishi *et al.*, 1984; Maraguchi *et al.*, 1985; Nakagawa *et al.*, 1985; Nakamura *et al.*, 1984; Leibson *et al.*, 1984; Sidman *et al.*, 1984). The role of BSF-1 in this model is primarily based on data obtained from murine studies utilizing anti-Ig. It can be seen in one postulated pathway that anti-Ig is thought to stimulate B cell entry into early G_1. Progression into the initial S phase is then thought to require the presence of BCGF and IL-1 acting as a cofactor. BSF-1 appears to be active on resting B cells before the signal delivered by anti-Ig. This initial signal is thought to be important in preparing the B cells to respond subsequently to anti-Ig. BCGF-II is shown to support the growth of dextran sulfate-stimulated B cells and of cells that are already activated. As shown in Fig. 8, lymphokines with reported differentiative effects are primarily thought to act after the proliferative phase.

Much less investigation into the temporal requirements of various lymphokines has been carried out in the human system. Studies in the human system that have examined this issue have shown that the presence of T cell factors during initial activation of peripheral blood B cells with SA did not result in an increase in the total number of activated cells but did result in the delivery of a predivisional differentiative signal to some of the B cells that resulted in significant enhancement in the subsequent generation of Ig-secreting cells upon culture with T cell-derived lymphokines (Jelinek and Lipsky, 1985). This finding implies that the model shown in Fig. 8 must be reconsidered with the possibility that differentiative influences also might play a role before B cell proliferation.

In order to understand the sequence of T cell signals involved in the activation, proliferation, and differentiation of human B cells, experiments with purified recombinant lymphokines were undertaken (Jelinek *et al.*, 1986b). The effect(s) of r-IL-2 and r-IFN-γ on SA-stimulated B cells was examined in detail. The effects of these lymphokines were examined on both highly purified, monocyte, and T cell-depleted B cells (see Jelinek and Lipsky, 1985) as well as on B cells completely depleted of any contaminating cells by negative selection on the fluorescence-activated cell sorter following staining with a cocktail of T cell and Mϕ-specific monoclonal antibodies. When B cell DNA synthesis was examined (Table IX), T cell supernatants and r-IL-2 were found to augment DNA synthesis of both SA-stimulated standard and FACS-selected B cells. In some experiments,

TABLE IX

r-IL-2 BUT NOT r-IFN-γ PROMOTES DNA SYNTHESIS AND DIFFERENTIATION INTO Ig-SECRETING CELLS OF SA-STIMULATED B CELLS[a]

Assay	Experiment	Cells	Lymphokine	Mitogen: None	Mitogen: *S. aureus*
				[^{3}H]Thymidine incorporation (cpm × 10^{-3})	
B cell DNA synthesis	1	Control	0	0.7	0.7
			T supt	0.5	17.6
			r-IL-2	0.5	12.0
			r-IFN-γ	0.1	0.5
			r-IL-2 + r-IFN-γ	0.7	12.5
	2	Control	0	0.1	1.1
			T supt	1.3	25.2
			r-IL-2	2.6	29.2
		FACS selected	0	0.2	1.4
			T supt	1.4	25.3
			r-IL-2	1.8	30.3
				(ISC per 10^6 B cells × 10^{-3})	
Generation of Ig-secreting cells	1	Control	0	0	0
			T supt	1.0	75.6
			r-IL-2	0	78.2
			r-IFN-γ	0	0
			r-IL-2 + r-IFN-γ	0	66.6
	2	Control	0	0	0.2
			T supt	4.0	144.0
			r-IL-2	7.8	185.6
		FACS selected	0	0	0
			T supt	6.8	174.4
			r-IL-2	11.8	296.0

[a] Control and FACS-selected (OKT3, OKT11 negative) B cells (2.5 × 10^4/well) were cultured alone or with SA in the presence or absence of T cell supernatant, 100 U/ml r-IL-2, or 100 U/ml r-IFN-γ. [^{3}H]Thymidine incorporation and the number of Ig-secreting cells (ISC) generated were assayed on day 5. From Jelinek *et al.* (1986a).

T cell supernatant or r-IL-2 also stimulated modest B cell DNA synthesis in the absence of SA. By contrast, r-IFN-γ did not promote B cell DNA synthesis in the presence or absence of SA. In similar experiments, both T cell supernatant and r-IL-2, but not r-IFN-γ, were found to support the generation of Ig-secreting cells from SA-stimulated standard and FACS-selected B cells (Table IX). Additional ex-

TABLE X
THE ACTION OF IL-2 IN CULTURES OF B CELLS ACTIVATED WITH SA OR SA + T CELL SUPERNATANT[a]

Assay	Cells	First incubation stimulus[b]	Addition during second incubation		
			Nil	T supt	IL-2
			(ISC per 10^6 B cells $\times 10^{-3}$)		
A. ISC generation[c]	Control	SA	0.4	27.6	3.2
		SA + T supt	0.6	30.0	28.2
	FACS selected	SA	1.0	21.4	1.4
		SA + T supt	1.8	39.8	41.6
			[^{3}H]Thymidine incorporation (cpm $\times 10^{-3}$)		
B. DNA synthesis	Control	SA	1.0	10.0	3.5
		SA + T supt	1.6	10.0	11.7
	FACS selected	SA	0.8	7.9	3.4
		SA + T supt	1.2	13.7	10.2

[a] From Jelinek *et al.* (1986b).

[b] Control or FACS-selected (OKT3, T11, 63D3, HNK-1 negative) B cells were cultured with SA with or without T cell supernatant for 48 hr. Afterward, the cells were washed three times and recultured with or without T cell supernatant, or 100 U/ml IL-2 (Collaborative Research).

[c] Ig-secreting cells and [^{3}H]thymidine incorporation were quantitated on day 3 of second incubation.

periments demonstrated that both proliferation and differentiation of SA-stimulated B cells were observed with concentrations of r-IL-2 as small as 1 U/ml. Moreover, anti-Tac (kindly provided by Dr. Thomas Waldmann), a monoclonal antibody directed at the binding site of the IL-2 receptor, completely prevented proliferation and differentiation of SA-activated B cells supported by r-IL-2 (Jelinek and Lipsky, unpublished observations).

B cells activated with SA for 48 hours generate large numbers of Ig-secreting cells upon subsequent incubation with T cell supernatant (Jelinek and Lipsky, 1985) (Table X). When IL-2 was examined for its ability to function in a similar capacity, it was found that this lymphokine was much less effective than T cell supernatant at supporting the generation of Ig-secreting cells from B cells activated by SA alone. Substantial numbers of Ig-secreting cells were generated in response to IL-2 only when B cells had been initially activated with SA and T

cell supernatant. Similar results were obtained when B cell DNA synthesis was examined. The effects of IL-2 were comparable when highly purified FACS-selected B cells were employed as responders, thus confirming that IL-2 was exerting its effect directly on the B cell. It was also shown that the differences in responsiveness to r-IL-2 by SA or SA + T cell supernatant-activated B cells did not result simply from differences in the concentration of r-IL-2 necessary to yield optimal stimulation as concentrations of r-IL-2 as large as 500 U/ml did not support responses of SA-activated cells as effectively as T cell supernatants (Jelinek *et al.*, 1986b).

Each of the aforementioned experiments demonstrated that T cell supernatant was much more effective than r-IL-2 at supporting SA-activated B cell responses. It was of interest to determine whether the difference between the capacity of r-IL-2 and T cell supernatant to promote responsiveness of SA-activated B cells could be accounted for by IFN-γ in the T cell supernatant. As shown in Table XI, T cell supernatant was much more effective than r-IL-2 at promoting DNA synthesis and generation of ISC from SA-activated B cells. The addition of r-IFN-γ to r-IL-2-supported cultures of SA-activated B cells enhanced the generation of ISC but not DNA synthesis. By contrast, the addition of r-IFN-γ had no effect on responsiveness of B cells initially activated by SA + T cell supernatant. It should be noted that the generation of ISC from SA-activated B cells supported by the

TABLE XI
EFFECT OF THE PRESENCE OF IFN-γ ON THE IL-2 RESPONSIVENESS OF SA-ACTIVATED B CELLS[a]

Assay	First incubation[b]	Addition during second incubation			
		Nil	T supt	r-IL-2	r-IL-2 + r-IFN-γ
		(ISC per 10^6 B cells × 10^{-3})			
ISC generation	SA	5.6	24.0	7.2	13.6
	SA + T supt	3.4	34.4	37.6	25.8
		(cpm × 10^{-3})			
DNA synthesis	SA	1.2	7.9	4.8	5.1
	SA + T supt	2.0	16.1	11.9	11.5

[a] From Jelinek *et al.* (1986b).

[b] FACS-selected T3, T11-negative B cells were cultured with SA or SA + T cell supernatant. Cells were then washed and incubated for an additional 72 hr with T cell supernatant, 50 U r-IL-2/ml, or 50 U r-IL-2/ml plus 100 U/ml r-IFN-γ before assaying for DNA synthesis and the generation of Ig-secreting cells.

combination of r-IL-2 and r-IFN-γ was still small in comparison to the responses supported by T cell supernatant. These data therefore suggest that the presence of IFN-γ does not explain the difference in the magnitude of responses of SA-activated B cells supported by T cell supernatant and r-IL-2. Rather, the data supported the conclusion that a B cell stimulatory factor(s) in T cell supernatant that is distinct from IL-2, IFN-γ, or the combination provides this activity.

It was clear that the presence of T cell supernatant during the initial activation with SA induced subsequent B cell responsiveness to IL-2. Since the T cell supernatant employed contained a number of activities, including IL-2 (approximately 200 U/ml; Jelinek and Lipsky, unpublished observations) and IFN-γ as well as BCGF and BCDF-like activities, it was possible that one or another of these factors accounted for subsequent IL-2 responsiveness. In Table XII it can again be seen that the inclusion of T cell supernatant during the preincubation of B cells with SA resulted in markedly increased subsequent generation of Ig-secreting cells in response to r-IL-2. The inclusion of either r-IL-2 or r-IFN-γ instead of T cell supernatant during the initial activation with SA also resulted in enhanced subsequent responsiveness to either T cell supernatant or r-IL-2. Although r-IFN-γ was also active during the first incubation with SA in augmenting subsequent B cell responsiveness to T cell supernatant or r-IL-2, it

TABLE XII
B CELLS INITIALLY ACTIVATED WITH SA IN THE PRESENCE OF r-IL-2 OR r-IFN-γ ARE ENHANCED IN SUBSEQUENT RESPONSIVENESS TO r-IL-2[a]

First incubation stimulus	ISC per 10^6 B cells ($\times 10^{-3}$)			
	Addition during second incubation			
	Nil	T supt	r-IL-2	r-IFN-γ
Nil	0	29.4	0.4	0
SA	0	205.0	50.0	0
SA + T supt	1.4	238.0	284.2	2.4
SA + r-IL-2	0.8	260.4	187.2	0.8
SA + r-IFN-γ	0	255.6	137.2	0.8

[a] B cells were preincubated as described in the legend to Table X in the presence of various combinations of SA, T cell supernatant, 100 U/ml r-IL-2, or 100 U/ml r-IFN-γ. ISC were quantitated on day 3 of the second incubation. Adapted from Jelinek *et al.* (1986b).

was somewhat less active than r-IL-2. r-IFN-γ, however, was unable to promote differentiation of activated B cells into Ig-secreting cells during the terminal incubation even when T cell supernatant had been present during the initial activation period. By contrast, r-IL-2 was not only effective at promoting the generation of Ig-secreting cells following activation with SA + T cell supernatant but it also supported the generation of Ig-secreting cells from B cells initially activated with SA + r-IL-2, thus indicating that IL-2 alone could function as a B cell differentiation factor. It was additionally demonstrated that although r-IL-2 promoted modest growth of SA-activated B cells, markedly enhanced B cell DNA synthesis and growth was noted when the cells had been initially activated by SA in the presence of T cell supernatant or r-IL-2 (Jelinek *et al.*, 1986b).

The studies on the temporal relationship of these various signals to the triggering event mediated by SA have demonstrated that (1) T cell-derived lymphokines are not required during initial activation triggered by optimal concentrations of SA; (2) the major requirement for growth-promoting activity appears to occur after initial division; and (3) a B cell differentiation signal received before B cells have entered the initial S phase may be delivered by T cell supernatant, IL-2, or IFN-γ. Although the enhancing effect of this signal could be demonstrated even when crude T cell supernatants were used to promote responses of the activated B cells, it was most apparent when proliferation and differentiation of the activated B cells were driven by IL-2. Growth and differentiation signals conveyed after initial cell division induced by SA alone were most effectively delivered by T cell supernatant but not IL-2, IFN-γ, or the combination of IL-2 and IFN-γ.

These findings suggest a somewhat different view of the role of T cell-derived lymphokines in B cell activation. A model incorporating these findings is shown in Fig. 9. The results suggest that there are two pathways of B cell activation. One pathway requires T cell influences from initial triggering to allow subsequent maximal proliferation and differentiation in response to IL-2. The alternative pathway in which B cells are activated in the absence of T cell influences, subsequently requires a growth and differentiation promoting activity(s) within T cell supernatant that is distinct from IL-2. This activity in T cell supernatant may be representative of classically defined BCGF or BCDF, i.e., the activities that support the growth of activated B cells. However, when a commercially purified preparation of the low-molecular-weight form of BCGF was examined, it was found to be unable to promote responsiveness of SA-activated B cells to IL-2

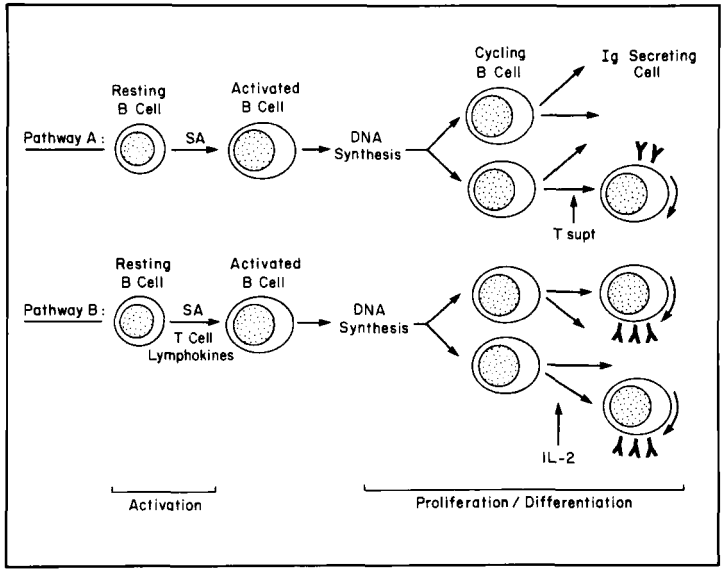

FIG. 9. Proposed model of the role of the various lymphokines in human B cell activation.

(Jelinek and Lipsky, unpublished observations). It remains possible, therefore, that the high-molecular-weight form of BCGF (BCGF-II) is the functionally active lymphokine in this regard.

The early requirement for T cell influences to promote maximal IL-2-driven B cell responses could have many explanations. Although T cell influences are not required for the expression of IL-2 receptors, they may be necessary for B cells to express high-avidity IL-2 receptors. Recent work by Prakash *et al.* (1985) has indicated that high-affinity IL-2 receptors were induced on murine B cells only when stimulated by both anti-Ig and T cell-derived helper factors. If human B cell responses to IL-2 require high-affinity IL-2 receptors and the induction of high-affinity IL-2 receptors requires T cell lymphokines, then proliferation and differentiation of B cells in response to IL-2 would be anticipated to require the presence of T cell influences during initial activation. It has been suggested that T cell expression of IL-2 receptors is dependent on exogenous IL-2, suggesting that IL-2 might induce its own receptor (Welte *et al.*, 1984). Moreover, it has recently been shown that IL-2 can directly upregulate both transcription of the IL-2 receptor gene (Depper *et al.*, 1985) and also the expression of high-affinity IL-2 receptors on thymocytes (Reem *et al.*, 1985). Although these results support the possibility that the role of T

cell lymphocytes in initial B cell activation involves the induction of high-avidity IL-2 receptors, there is controversy concerning the effect of IL-2 on the expression of high-avidity IL-2 receptors. Thus, Smith and Cantrell (1985) have suggested that IL-2 down-regulates high-affinity IL-2 receptors on activated T cells. It remains possible, therefore, that the early presence of T cell influences such as IL-2 and IFN-γ during B cell activation delivers a maturational signal that is distinct from their effect on the expression of high-affinity IL-2 receptors.

The state of activation or maturation of individual B cell precursors may determine the pathway of activation necessary for optimal stimulation. In this regard, we have found that FACS-selected IgD^- peripheral blood B cell responses are supported to a much greater extent by IL-2 than are the IgD^+ B cells (Table XIII). We have previously shown that the IgD^- B cells obtained from peripheral blood largely represent postswitch memory B cells (Jelinek *et al.*, 1986a). It is interesting to speculate that the IgD^- B cells are more responsive to IL-2 because of their state of maturation. In this regard, it has been suggested by Mond and colleagues (1985) that IL-2 responsiveness by murine B cells is limited to B cells that may have been activated by an initial linked interaction with T cells.

A number of inconsistencies remain to be resolved with regard to the action of IL-2 on B cells. One of these involves the role of IL-2 in supporting responses of anti-immunoglobulin-stimulated B cells. Anti-Ig-stimulated B cells express IL-2 receptors as detected with the anti-Tac monoclonal antibody (Muraguchi *et al.*, 1985; Suzuki and Cooper, 1985; Boyd *et al.*, 1985b). Moreover, Suzuki and Cooper (1985) demonstrated that mitogenic doses of a monoclonal anti-IgM antibody activated resting human peripheral blood B cells to express IL-2 receptors and that IL-2 greatly augmented their proliferative response to anti-IgM. By contrast, it has been reported that IL-2 may not significantly augment anti-Ig-induced proliferation of murine spleen B cells (Parker, 1982; Mond *et al.*, 1985) or human tonsil or spleen B cells (Jung *et al.*, 1984; Nakagawa *et al.*, 1985; Schwarting *et al.*, 1985) although crude T cell supernatants could promote B cell DNA synthesis. This suggests the possibility that B cells obtained from different anatomic sites may vary in their lymphokine requirements for optimum responsiveness. Experiments employing SA as a polyclonal B cell activator, however, have indicated that proliferation of peripheral blood (Tsudo *et al.*, 1984; Nakagawa *et al.*, 1985; Jelinek *et al.*, 1986b), tonsil (Muraguchi *et al.*, 1985; Teranishi *et al.*, 1984), or spleen (Mingari *et al.*, 1985) B cells may be augmented by IL-2. These results suggest that the nature of the activation signal may also play a

TABLE XIII
ABILITY OF IL-2 TO SUPPORT RESPONSES OF IgD^+ AND IgD^- B CELLS[a]

Population	Lymphokine	Mitogen: None	Mitogen: *S. aureus*
		[^{3}H]Thymidine incorporation (cpm × 10^{-3})	
Control	0	0.1	0.6
	T supt	1.2	20.3
	r-IL-2	0.2	6.8
IgD^+	0	0.1	0.5
	T supt	1.1	20.9
	r-IL-2	0.1	3.7
IgD^-	0	0.1	0.4
	T supt	2.5	21.8
	r-IL-2	0.6	16.5
		ISC per 10^3 B cells	
Control	0	1.0	0
	T supt	12.2	132.5
	r-IL-2	0.5	30.0
IgD^+	0	0	0.2
	T supt	0.5	73.8
	r-IL-2	0	17.5
IgD^-	0	0.2	0.5
	T supt	11.8	161.5
	r-IL-2	0.2	192.0

[a] Control unsorted B cells and B cells sorted into fluorescence-positive subsets after reacting with anti-IgD and fluorescence-negative subsets after reacting with anti-IgD, OKT3, OKT11, HNK-1, and 63D3, were cultured alone or with SA in the presence or absence of T cell supernatant or 100 U/ml r-IL-2. The generation of Ig-secreting cells and [^{3}H]thymidine incorporation were assayed on day 5.

role in determining the lymphokine requirement for B cell responsiveness. Apparent stimulus-related induction of responsiveness to T cell lymphokines has also been observed in the mouse, where it has been reported that neither IL-2 nor crude T cell supernatants could support maximal proliferation of B cells stimulated with lipopolysaccharide or anti-Ig alone (Zubler *et al.*, 1984), whereas either could

support optimal responses of B cells stimulated with LPS and anti-Ig together (Zubler *et al.*, 1984; Zubler, 1984).

In order to probe the lymphokine requirements of various B cell populations, responsiveness of human peripheral blood, spleen, and lymph node B cells were examined. When anti-IgM and SA and various lymphokines were employed to stimulate the three populations of highly purified B cells, differences were noted in the response patterns (Jelinek and Lipsky, 1987). DNA synthesis by peripheral blood B cells stimulated with soluble $F(ab')_2$ fragments of goat anti-IgM heavy chain specific antibody (anti-μ), or SA, was supported by r-IL-2 and T cell supernatant (Table XIV). The overall magnitude of anti-μ-stimulated responses, however, was considerably lower than SA-stimulated responses. When spleen B cells were examined (Table XV), r-IL-2 augmented proliferation of SA-activated B cells just as effectively as T cell supernatant. In contrast to peripheral blood B cells, r-IL-2 was only minimally able to support DNA synthesis by anti-μ-activated spleen B cells, whereas T cell supernatant supported substantial DNA synthesis. Analysis of lymph node B cell responses revealed that T cell supernatant supported considerable DNA synthesis by SA and anti-μ-stimulated lymph node B cells but IL-2 was considerably less effective in supporting responses by either of the stimuli.

These results suggest that the disparity in responsiveness observed between B cells obtained from different anatomic sites resulted not only from differences in intrinsic responsiveness and lymphokine re-

TABLE XIV
COMPARISON OF THE ABILITY OF T CELL SUPERNATANT AND IL-2 TO SUPPORT RESPONSES OF PERIPHERAL BLOOD B CELLS[a]

	[^{3}H]Thymidine incorporation (cpm × 10^{-3})		
	Stimulus		
Lymphokine	Nil	*S. aureus*	Anti-μ
Nil	0.4 ± 0.1	2.0 ± 0.5	0.3 ± 0.1
T cell supernatant	2.2 ± 0.3	28.8 ± 3.8	6.6 ± 1.1
r-IL-2	2.7 ± 0.3	28.0 ± 3.2	6.8 ± 0.8

[a] Peripheral blood B cells were cultured alone, with SA, or with 10 μg/ml $F(ab')_2$ anti-μ antibody in the presence or absence of T cell supernatant or 50 U/ml r-IL-2. [^{3}H]Thymidine incorporation was assayed on day 5. Data represent mean ± SEM of four experiments. From Jelinek and Lipsky (1987).

quirements but also from the nature of the stimuli used in activating the B cells. In support of this concept, Swain and colleagues (1983) have demonstrated that murine B cells stimulated with anti-IgM were augmented in growth by B cell stimulatory factor-1 (BSF-1) but not by BCGF II. Conversely, B cells stimulated with dextran sulfate proliferated in response to BCGF II but not by BSF-1 (reviewed by Howard *et al.*, 1984). In addition, human BSF-1 has been shown to support the proliferation of anti-IgM- but not SA-activated human tonsillar B cells (Yokata *et al.*, 1986). IL-2 may therefore also behave in a stimulus-dependent manner when human spleen and lymph node B cells are examined instead of peripheral blood B cells (Jelinek and Lipsky, 1987).

In contrast to the ability of T cell supernatant to support B cell proliferation of the B cell populations activated with SA, IL-2 alone was much less efficient than T cell supernatant at supporting proliferation of anti-μ-activated spleen and lymph node B cells (Table XVI). However, when a commercially available preparation of low-molecular-weight BCGF was added to the IL-2-supported cultures, synergistic amplification of responsiveness allowed maximal DNA synthesis. A number of other studies have demonstrated synergism between various helper factors in promoting B cell responses (Howard *et al.*, 1984; Yoshizaki *et al.*, 1983; Murray *et al.*, 1985). It is possible that this

TABLE XV
COMPARISON OF THE ABILITY OF T CELL SUPERNATANT AND IL-2 TO SUPPORT RESPONSES OF HUMAN B CELLS[a]

		[^{3}H]Thymidine incorporation (cpm $\times 10^{-3}$)		
		Stimulus		
B cell source	Lymphokine	Nil	*S. aureus*	Anti-μ
Spleen	Nil	0.1 ± 0.0	2.6 ± 0.1	0.1 ± 0.1
	T supt	0.7 ± 0.1	39.8 ± 2.3	18.6 ± 1.0
	r-IL-2	0.2 ± 0.1	45.6 ± 2.5	2.6 ± 0.5
Lymph node	Nil	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
	T supt	1.2 ± 0.0	12.0 ± 1.7	10.2 ± 0.2
	r-IL-2	0.3 ± 0.0	3.0 ± 0.1	0.8 ± 0.1

[a] Spleen or lymph node B cells were cultured alone, with SA, or with 10 μg/ml F(ab')$_2$ anti-μ antibody in the presence or absence of T cell supernatant or 100 U/ml r-IL-2. [^{3}H]Thymidine incorporation was assayed on day 5. Adapted from Jelinek and Lipsky (1987).

requirement for more than one lymphokine to support spleen and lymph node responsiveness reflects the variability in subset composition of the B cells obtained from different anatomic locations. We (Jelinek *et al.*, 1986a) as well as other investigators (Nakagawa *et al.*, 1985; Anderson *et al.*, 1985; Waldschmidt *et al.*, 1985) have demonstrated the variability in responsiveness observed when different subsets of B cells were analyzed. When SA was used to activate B cells, less of a disparity in the ability of IL-2 to support responses was noted between the B cell populations.

When the three populations of B cells were compared for the ability of IL-2 to support Ig-secreting cell generation following activation with SA, a disparity in response patterns was again noted. As shown in Table XVII, T cell supernatant and r-IL-2 were able to support the differentiation of ISC from peripheral blood and spleen B cells comparably. By contrast, the number of Ig-secreting cells generated from lymph node B cells supported by r-IL-2 was only 10–15% as large as that supported by T cell supernatant. These results emphasize the importance of both the activating stimulus and the origin of the B cells in determining the lymphokine requirements of human B cell responsiveness.

TABLE XVI
EFFECT OF "BCGF" ON THE IL-2 RESPONSIVENESS OF ACTIVATED B CELLS[a]

		[^{3}H]Thymidine incorporation (cpm × 10^{-3})		
		Stimulus		
B cell source	Lymphokine	Nil	*S. aureus*	Anti-μ
Spleen	Nil	0.1 ± 0.0	1.2 ± 0.1	0.1 ± 0.0
	T supt	0.8 ± 0.1	25.4 ± 2.6	18.1 ± 0.9
	r-IL-2	0.8 ± 0.1	28.8 ± 1.1	4.6 ± 0.5
	"BCGF"	1.4 ± 0.0	4.7 ± 0.2	3.7 ± 0.2
	r-IL-2 + "BCGF"	2.5 ± 0.3	38.3 ± 0.4	23.9 ± 1.4
Lymph node	Nil	0.0 ± 0.0	0.9 ± 0.1	0.2 ± 0.0
	T supt	0.2 ± 0.0	27.9 ± 3.7	1.2 ± 0.3
	r-IL-2	0.1 ± 0.0	7.0 ± 1.0	0.1 ± 0.0
	"BCGF"	0.4 ± 0.0	6.3 ± 0.5	0.4 ± 0.0
	r-IL-2 + "BCGF"	1.1 ± 0.2	24.3 ± 1.1	5.7 ± 0.9

[a] Spleen, lymph node, or peripheral blood B cells were cultured alone, with SA, or with F(ab')$_2$ anti-μ antibody in the presence or absence of T cell supernatant, 100 U/ml r-IL-2, 20% "BCGF," or the combination of r-IL-2 and BCGF. [^{3}]Thymidine incorporation was assayed on day 5. Adapted from Jelinek and Lipsky (1987).

TABLE XVII
CAPACITY OF T SUPERNATANT AND IL-2 TO SUPPORT DIFFERENTIATION OF SA-STIMULATED SPLEEN AND LYMPH NODE B CELLS[a]

		ISC per 10^6 B cells ($\times 10^{-3}$)	
		Stimulus	
B cell source	Lymphokine	None	*S. aureus*
Peripheral blood	Nil	0	0
	T supt	1.0	230.4
	r-IL-2	0.2	214.8
Spleen	Nil	0	0
	T supt	0.2	316.8
	r-IL-2	0	473.6
Lymph node	Nil	0	0
	T supt	0	169.6
	r-IL-2	0	23.0

[a] Peripheral blood, spleen, and lymph node B cells were cultured alone or with SA in the presence or absence of T cell supernatant or 100 U/ml r-IL-2. Ig-secreting cells were assayed on day 5. Adapted from Jelinek and Lipsky (1987).

VI. Concluding Remarks

This article has reviewed a number of aspects of human B cell activation, proliferation, and differentiation. The signals involved in initial activation of B cells and their impact on subsequent proliferation and differentiation were discussed. Two aspects of this process were delineated in detail. First, it was apparent that the nature of the signals transmitted during initial activation determined the capacity of the activated cells to respond subsequently with ongoing proliferation and differentiation. Second, the relationship between proliferation and differentiation of B cells was found to be far more complex than anticipated with cell division not only noted to be a prerequisite for Ig secretion but also proliferation of the Ig-secreting cells themselves was found to be necessary for subsequent propagation and amplification of the response. Additional complexity in the regulation of B cell responses was noted when the responsiveness of various B cell populations and subpopulations was examined. Anatomic site of origin, surface isotype expression, and activation status were all found to play a role in determining the nature of the activation requirements and response potential of B cell subsets. Finally, the roles of various

cytokines in the regulation of B cell responses were explored. IL-2, IFN-γ, BCGF, and IL-1 were all found to regulate B cell function. Moreover, specific cytokines were documented to provide signals both during and after activation that governed the nature and magnitude of the resultant response. It is clear that regulation of human B cell responsiveness is an exquisitely controlled and remarkably complex process. The availability of well-defined pure lymphokines, monoclonal antibodies to isolate subpopulations, and techniques to assay a variety of responses has permitted a better appreciation of the regulation of human B cell function and should permit rapid development of additional insight into this complex process.

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Biological Activities Residing in the Fc Region of Immunoglobulin

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I. Introduction

Fc receptors (FcR)[1] are present on a number of cell types, including those of the immune system, and have been implicated in the regulation of immune reactivity. They have been associated with (1) the release of biologically active mediators, (2) autoimmune disorders, and (3) regulation of lymphocyte function through their interaction with antibody complexed to antigen. Concerning this latter point, it has been established that immune complexes (IC), largely via the Fc region, can contribute to either the enhancement and/or suppression of humoral and cell-mediated immunity depending upon (1) antigen–antibody ratio, (2) antibody isotype and subclass, (3) immune status of host or lymphocyte donor, and (4) presence or absence of the Fc portion of antibody. More recently, it has been suggested that the FcR may be involved in the focusing of antibody, in the form of an IC, on macrophages or monocytes, where these cells enzymatically cleave the Fc portion of antibody into biologically active immunoregulatory peptides (1).

In this article, we will summarize studies involving lymphocyte activation and immune regulation by peptides derived from IC and Fc fragments of immunoglobulin (Ig). Furthermore, the cellular and subcellular events involved in lymphocyte activation by these biologically active peptides will be defined and discussed.

A. Immunoglobulin Structure

There are five major classes of mammalian Ig, i.e., IgG, IgM, IgA, IgD, and IgE. The fundamental structure of the Ig molecule consists

[1] Abbreviations: AA, arachidonic acid; AHGG, aggregated human γ-globulin; BCAF, B cell activating factor; C, complement; CMI, cell-mediated immunity; CML, cell-mediated lympholysis; DTH, delayed type hypersensitivity; FcR, Fc receptor; IC, immune complex; Ig, immunoglobulin; IL-1, interleukin 1; IL-2, interleukin 2; IM, indomethacin; LT, leukotriene; PG, prostaglandin; RFIC, rheumatoid factor-associated immune complex.

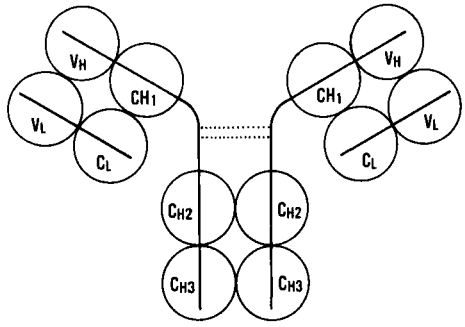

FIG. 1. Domain model for the structure of human IgG.

of two identical light (L) and two identical heavy (H) chains. Some secreted Ig classes have greater than one four-chain molecule, e.g., 19 S IgM consists of five four-chain units joined together through the J chain (2). Furthermore, secretory IgA contains an additional polypeptide chain, the secretory piece, which is responsible for its entry into secretory fluids. A detailed summary of the literature on Ig structure is beyond the scope of this article, and we are primarily concerned with IgG, therefore, selected references on the structure of IgG will be addressed. A more detailed analysis of Ig structure can be obtained in recent reviews (3–7).

The 7 S IgG molecule consists of 2 H and 2 L (2) chains covalently linked by interchain disulfide bonds. Each chain has an amino terminal part of approximately 110 amino acids which contains the variable region that is primarily involved in the specific interaction with antigen and a constant portion of approximately 110 amino acids for the L chain and approximately 330 amino acids for the H chain. The H chains are structurally and antigenically different for each Ig class. Edelman and Gall (8) proposed that the IgG molecule is folded into a series of domains (Fig. 1). The H chain consists of 4 domains of approximately 110 amino acid residues with regularly spaced intrachain disulfide bonds. One domain comprises the variable region, V_H, while the other domains are present in the constant regions, CH_1, CH_2, and CH_3. Data from primary sequence analysis have shown that the domains have a high degree of homology. X-Ray crystallographic analysis indicates that all antibodies of a given class have the same basic structure. Furthermore, these studies revealed that the IgG molecule is composed of β-pleated sheets stabilized by hydrogen bonds.

Structural analysis of IgG has also been carried out by the use of enzymatic cleavage techniques (Fig. 2). It was first demonstrated by Porter (9) that cleavage of rabbit IgG with papain at neutral pH re-

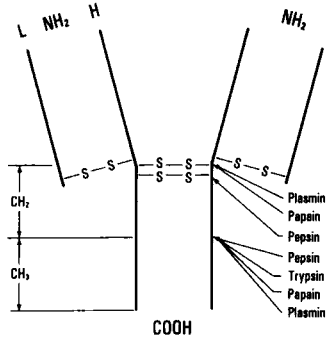

FIG. 2. Enzymatic cleavage of human IgG.

sulted in the production of two Fab fragments and one Fc fragment. Papain attacks the H chain in the hinge region at a site on the amino terminal side of the inter-H chain disulfide bonds. The Fab fragment consists of an L chain and the V_H and CH_1 portion of the H chain, the latter termed the Fd fragment. The Fc fragment consists of the disulfide-linked carboxyterminal portion of both H chains (CH_2 and CH_3). IgG can be cleaved into smaller fragments by a variety of enzymes other than papain. Pepsin has been shown to cleave Ig on the carboxy terminal side of the inter-H chain disulfide bonds, resulting in the formation of a large F $(ab')_2$ fragment (10). The Fc fragment is degraded by pepsin into heterogeneous peptides. One fragment, pFc', has been shown to correspond to approximately the CH_3 domain of the molecule, whereas the CH_2 domain is reduced to small peptides (11). Ellerson *et al.* (12) found that acid-treated Fc fragments derived from IgG_1 were cleaved at the junction between CH_2 and CH_3 domains by trypsin. CH_3-like fragments are also derived from IgG by cleavage with plasmin (13,14).

B. RECEPTORS FOR THE Fc REGION OF Ig (FcR)

1. Cellular Distribution

Binding of Ig to cell surface receptors has been the subject of intensive investigation. The binding is specific, dependent on the Fc portion of Ig, and, in some instances, multiple receptor specificities exist on a single cell type. FcR have been demonstrated by rosette formation, radiolabeled ligand binding, and inhibition studies on a variety of mammalian cells which include lymphocytes (15–24), monocytes/macrophages (25–31), platelets (32–34), neutrophils (35,36), eosinophils (37–39), basophils and mast cells (40–45), and placental tissue

(46,47). IgG, IgM, and IgA have been shown to bind via FcR to cell surfaces. Studies described below will focus on FcR for IgG. A more detailed account of FcR for all Ig classes can be obtained in a number of recent review articles (5,48,49).

a. Macrophages and Monocytes. The demonstration by Boyden and Sorkin (50,51) of cytophilic antibody for guinea pig macrophages was the first indication of cellular receptors for Ig. Berken and Benacerraf (52) characterized the antibody as IgG and showed that binding occurred through the Fc region. Similarly the Fc portion of IgG was shown to be a requisite for binding of IC to macrophages (53,54).

The binding of human IgG to human peripheral blood-derived monocytes was first described by Huber and Fudenberg (26). Monocytes were also found to bind erythrocytes sensitized with human IgG antibodies (55). The Fc portion of antibody was considered to be required because erythrocytes sensitized with Fab fragments of antibody were unable to bind. Furthermore, the FcR were found to be specific for IgG_1 and IgG_3 by rosette inhibition analysis using human IgG_1, IgG_2, IgG_3, and IgG_4 myeloma proteins (56). Similar results were obtained by Okafor *et al.* (57), who demonstrated that Fc fragments derived from human IgG_1 and IgG_3 inhibited rosette formation between monocytes and anti-Rh-coupled human erythrocytes. These findings were further supported by Fries *et al.* (58), who found that ^{125}I-labeled IgG_1 and IgG_3 bound with equal affinity to monocytes whereas IgG_2 and IgG_4 bound with much less affinity. In contrast to the findings with IgG_1 and IgG_3, human IgM-coated erythrocytes were unable to form rosettes with human monocytes unless complement (C) was also added to the reaction, indicating a role for complement receptors (CR) in IC binding (59).

In addition to peripheral blood monocytes, several human tumor cell lines of monocyte lineage bear FcR. The U937 cell line, derived from a patient with histocytic lymphoma, contains receptors for IgG_1 and IgG_3 and, to a lesser extent, IgG_4 (60). IgG_1 and IgG_3 appear to be equal in ability to bind to the U937 cell line. The promyelocytic leukemic cell line, HL-60, has also been shown to bind IgG_1 and IgG_3 (61).

In addition to intact IgG, Fc fragments bind to human monocytes (61,61) and human monocyte and mouse macrophage cell lines (63,64). Fc fragments derived from a human IgG_1 myeloma protein were found to bind to the mouse macrophage-like cell line $P388D_1$ (Fig. 3). These fragments bound specifically to a single class of receptor with a K_a value of $4 \times 10^6\ M^{-1}$ and to approximately 2.4×10^5 binding sites/cell. In a homologous system Raychaudhuri *et al.* (63)

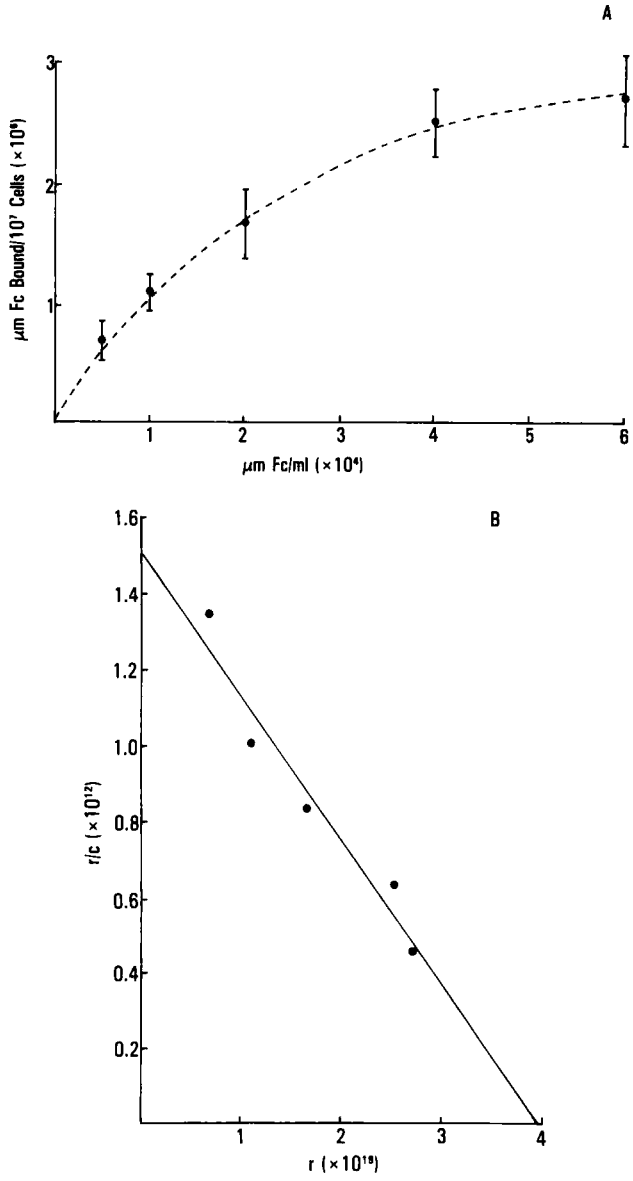

FIG. 3. (A) Binding of increasing amount of Fc fragments to P388D$_1$ cells. P388D$_1$ were incubated for 30 minutes with increasing amounts of ^{125}I Fc with or without excess unlabeled Fc. The data shown are corrected for nonspecific binding and each point represents the mean ± SE from five experiments. (B) Scatchard equation plot of data shown in (A). Reprinted from Hobbs *et al.* (1985). *Cell. Immunol.* **90**, 74.

observed that Fc fragments bound to the human cell lines U937, HL-60, and ML-1. Comparisons of binding of radiolabeled monomeric IgG and Fc fragments to U937 showed that these cells possess a single class of receptor for Fc and IgG. Fc fragments, however, were found to bind with a greater affinity ($K_{a_{Fc}} = 3.1 \times 10^9\ M^{-1}$ vs $K_{a_{IgG1}} = 0.98 \times 10^9\ M^{-1}$) than IgG.

It is well established that mouse macrophages possess FcR for more than one IgG subclass (64,65). The receptor which bound IgG_{2a}-sensitized erythrocytes was susceptible to cleavage by trypsin, whereas the IgG_{2b} site was resistant. These studies were later confirmed by Unkeless and Eisen (30) and Heusser *et al.* (66), who showed that trypsin-treated macrophages or macrophage cell lines were unable to bind IgG_{2a} but could still bind IgG_{2b}. The general consensus which arose from these experiments is that there are at least two types of FcR on mouse macrophages, one that is trypsin sensitive and binds mouse IgG_{2a}, and another one that binds monomeric IgG_{2b} and is trypsin resistant. Additional evidence for multiple receptors for the Fc portion of Ig comes from binding inhibition studies (66–69).

b. B Lymphocytes. Of primary importance in the implication of FcR in immune regulation was the finding of their presence on lymphocytes. Uhr and Phillips (53) originally reported that lymphocytes have a strong binding affinity for IC. Antigen–antibody complexes, aggregated IgG, and in some cases monomeric IgG have been shown to bind to lymphocytes by rosette formation with antibody-coated erythrocytes, autoradiography, and immunofluorescence. Furthermore, binding was observed with an intact Ig molecule but not Fab fragments, suggesting that binding occurred through the FcR (23,24,70,71).

B lymphocytes have been shown to bind Ig of the IgG (15,23,70,71), IgM (72,73), and the IgA classes (70,74). Binding of IgG to FcR on B lymphocytes appears to be dependent upon the IgG subclass and degree of aggregation. Aggregated IgG_1 and IgG_3 myeloma proteins bind more avidly to human B cells than aggregated IgG_2 and IgG_4 proteins (18,75). The relative ability of mouse IgG subclasses to bind to mouse B lymphocytes does not appear to be as clear cut. Some studies indicate that IgG_1, IgG_{2a}, and IgG_{2b} bind with equal affinity (70), while others report that IgG_{2a} and IgG_{2b} (16) or IgG_1 (24) bind the best.

The conformation of aggregated IgG apparently dictates to some extent the degree of binding to the FcR. Dickler and Kunkel (71) observed that aggregates of large sizes were optimal in binding to FcR. Anderson and Grey (16) reported that aggregated myeloma IgG

proteins of 100 molecules were optimal for binding to mouse spleen cells. Segal and colleagues (76,77), using chemically cross-linked rabbit IgG, observed that the binding affinity for trimers was stronger than that of dimers, and dimers bound more efficiently than monomers. However, as the size of the oligomer was further increased, the binding ability decreased.

c. T Lymphocytes. T lymphocytes obtained from both mice and humans also possess receptors for the Fc portion of IgG and IgM (16,78–84). in most instances, FcR for IgM and IgG on T cells have been detected by rosette formation with bovine erythrocytes coated with purified rabbit IgM or IgG. FcR for IgG can be detected on freshly isolated human T cells, whereas in order to detect receptors for IgM on T lymphocytes they must first be incubated *in vitro* in serum-free medium (82,85). FcR for IgM are most likely present on freshly isolated T cells but masked by serum IgM. Inhibition experiments employing Fc fragments derived from IgM and IgG reveal that FcR for IgM are specific for the Fc region of IgM (86,87) and that monomeric IgM binds better than pentameric IgM (88). As with FcR for IgG_{2a} and IgG_{2b}, FcR for IgM and IgG can be differentiated by their susceptibility to enzyme digestion. FcR for IgM have been shown to be susceptible to proteolysis, whereas FcR for IgG are relatively resistant (85). Although originally thought to represent markers for functional human T cell subsets, recent experiments (89) indicate that FcR for IgM and IgG are not stable surface structures but may be present on T cells only during certain stages of activation.

Murine T cells have also been shown to bind Ig. Basten *et al.* (90) reported that 25% of mouse peripheral lymphocytes with T cell markers contained FcR as detected by aggregated Ig and antigen–antibody complex binding. Stout and Herzenberg (80) reported that 23% of T cells from spleen and 10% of thymocytes are FcR positive. It appears that the affinity of binding of Ig to B cells is greater than to T cells, since radiolabeled aggregated Ig is able to kill B cells but not T cells (91), and large but not small aggregates bind significantly to T cells (16). Evidence exists which shows that mouse T cells bind to all subclasses of IgG but differ in their relative binding affinity. IgG_{2a} and IgG_{2b} have been shown to bind more avidly than IgG_1 to normal T cells (16,92,93). Binding of IgM to mouse T cells has also been reported by a number of investigators (94–96), however, others (80,97,98) have been unable to substantiate these results. Recently, Coico *et al.* (99) reported that mouse T cells bind IgD. These authors also observed that exposure to IgD *in vivo* or *in vitro* significantly

increased the number of IgD FcR^+ T cells in the spleen and lymph nodes but not in the thymus.

d. Platelets. Human platelets bear surface receptors for the Fc portion of IgG (32–34). Employing large numbers of myeloma proteins, Henson and Spiegelberg (34) observed no significant binding differences among IgG subclasses. Interaction of human platelets with IC (100,101) and aggregated myeloma proteins (33,34) results in platelet aggregation and the release of biological mediators such as vasoactive amines. As with macrophages and lymphocytes, the portion of the IgG molecule responsible for platelet activation is the Fc region since Fc fragments but not F $(ab')_2$ fragments induce serotonin release (34).

e. Neutrophils. Human neutrophils have been shown to bind to all subclasses of IgG (35,36), albeit IgG_1 and IgG_3 were found to bind better than IgG_2 and IgG_4. Both aggregated IgG and IgA induced neutrophils to release lysosomal enzymes whereas IgM, IgD, and IgE were all inactive (5).

f. Basophils and Mast Cells. Basophils and mast cells from humans and animals possess high-affinity receptors for the Fc portion of IgE (5,102–107). In addition to IgE, basophils and mast cells from rats bind IgG_1, but its affinity for the receptor is less than for IgE (108).

2. *Characterization of FcR for Ig*

a. Localization of FcR Binding Sites in the Fc Region. A frequently asked question has been where on the Fc portion of the IgG molecule is the region(s) which reacts with the Fc receptor. Although the Fc region of IgG is responsible for a wide variety of effector functions (4,49,50), the site involved for each of these activities may not be the same. Most binding studies have involved the use of Fc region subfragments, IgG deletion mutants, and synthetic Fc peptides (109,110). In general, experiments employing CH_2 domain fragments indicate that this region is not sufficient for FcR binding (7,110,111). However, CH_3 domain fragments have been shown to bind to FcR. Isolated CH_3 fragments coupled to sheep erythrocytes have been reported to form rosettes with macrophages (112,113). Ramasamy *et al.* (114) presented evidence that CH_3 region was required for reaction with lymphocyte FcR and observed that an IgG_1 mutant protein lacking the CH_3 domain failed to bind. Klein *et al.* (115) observed that the binding of human IgG_1 to mouse T cells resides in the CH_3 domain between residues 342–433. Additional support for the involvement of the CH_3 domain in FcR binding is derived from the observation of Barnett-Foster *et al.* (11), who reported that CH_3 fragments bind to receptors on human monocytes. Similar results were also obtained by Menta

and Painter (116) who observed that heterologous passive cutaneous anaphylaxis, mediated by mast cell binding of IgG_{2a}, also occurred with CH_3 domain fragments. In most of the above studies, however, higher molar concentrations of proteolytic fragments were needed to obtain binding levels similar to those using intact IgG, suggesting that the CH_2 domain contributes to the binding. An additional problem encountered when using enzymatic fragments of the Fc region was revealed by circular dichroism studies of Fc fragments, which showed that the sum of the conformations of isolated CH_2 and CH_3 domain fragments did not equal the conformation of the intact Fc fragment (117). These results suggest that the inability of CH_2 or CH_3 domain fragments to bind FcR in a manner equal to intact Fc fragments may be the result of a conformational change in the isolated domains. An alternate possibility is that FcR interacts with structure near the CH_2–CH_3 juncture, and enzymatic cleavage of the domain would affect this binding site. Based on the inferior binding of free CH_3 and the nonbinding of CH_2, it was proposed (111) that the binding site of the Fc region may either be composed of residues contributed by both the CH_2 and CH_3 domains or the CH_3-binding site is stabilized by the presence of the two CH_2 domains.

b. Physiochemical Characterization of the FcR for IgG. Physiochemical characterization of the IgG FcR has recently been afforded through the use of monoclonal antibody technology. Unkeless (118) first described the production of a monoclonal antibody, 2.4G2, secreted by a hybridoma derived from spleen cells of a rat immunized with the J774 and $P388D_1$ murine macrophage-like cell lines. Fab fragments from this monoclonal antibody were found to inhibit rosette formation by erythrocytes coupled with murine IgG_1 or IgG_{2b}, but not IgG_{2a}. Moreover, 2.4G2 did not compete with radiolabeled IgG_{2a} for FcR binding, thus suggesting a selective recognition for the trypsin-insensitive FcR (119). Although 2.4G2 was raised against mouse macrophage cell lines, it was found to bind to mouse lymphocytes which contained IgG FcR (118). FcR isolated from the J774 cell lysates by 2.4G2 affinity chromatography revealed two polypeptides of 60,000 and 47,000 M_r (49). Isolation of FcR from B cells, T cells, and macrophages revealed a significant M_r heterogeneity ranging from 45,000 to >70,000 M_r (49). Unkeless *et al.* (49) suggested that the range of M_r for FcR from different cell lineages could reflect proteolysis or altered glycosylation of the protein.

Isolation of FcR by other approaches basically agrees with the results obtained using the 2.4G2 monoclonal antibody. Loube and Dorrington (120) observed that internally labeled FcR derived from

the P388D$_1$ macrophage-like cell line consisted of 62,000 and 58,000 M_r species. Similar M_r species have been derived from rabbit alveolar macrophages (121), thioglycolate-elicited mouse peritoneal macrophages (122), and human mononuclear cells (123,124). These latter authors isolated a 60,000 M_r species from a surface-iodinated mixed cell population consisting of lymphocytes and monocytes.

II. Biological Properties of the Fc Region of Ig

Biological properties associated with antibody can be categorized into primary and secondary functions (5). Complexing of antibody with antigen through the Fab portion of the antibody molecule constitutes the primary function. Formation of antigen–antibody complexes also results in the appearance of secondary functions not associated with the free antibody molecule. These secondary biological properties are associated with the Fc region of the antibody molecule. Secondary biological properties of the Fc region of antibody include binding to C components and FcR, which results in cellular activation with release of biologically active mediators. In addition to these secondary activities, it is well established that antibody, via its Fc region, can have a variety of regulatory effects on the immune response.

A. Complement Fixation

Activation of the C cascade has been associated with the Fc portion of the antibody molecule. A detailed account of the literature describing C activation is beyond the scope of this article; therefore, only a summary of pertinent selected references will be addressed. A more detailed analysis can be obtained in recent reviews of the role of IC in C activation (5,109,125–127).

Complexes formed between antigen and antibody and Ig aggregates activate C by both the classical and alternate pathways (5,109,125–129). IC containing human IgG, IgM, or aggregated myeloma protein activate the classical pathway by combining with the C1q subunit of C1. C1q binding to IC results in a time-dependent change in C1q during which C1r and C1s bind to C1q, coverting C1s to C1-esterase. Formation of C1-esterase results in the activation of the remaining components of the C cascade (130). C1q, a 400,000 M_r glycoprotein (131), is a six-headed structure that resembles collagen (128,133–135). Binding of C1q to IgG occurs through the tulip head structures since enzymatic digestion of C1q destroys the heads, resulting in a molecule which is unable to bind Ig but can still interact with C1r and C1s (135–138).

Aggregated myeloma proteins of the IgG_1, IgG_2, and IgG_3 subclasses and IgM class are more efficient than aggregated IgG_4 in activating the classical pathway (5,139–141). Aggregated IgA, IgD, and IgE myeloma proteins do not appear to fix C by this pathway (142,143). C1q binding apparently resides in the CH_2 domain of IgG (110,144–146) and CH_4 domain of IgM (147,148). Yasmeen *et al.* (146) reported that when isolated CH_2 and CH_3 homology regions of human IgG were assessed for their ability to interact with C only CH_2 retained any activity. On a molar basis, the CH_2 fragment was less active than intact IgG in a standard C-fixation assay in which CH_2, Fc fragments, and intact IgG were absorbed as monolayers onto polystyrene beads. However, in a more sensitive fluid phase C1-binding assay, CH_2 showed the same binding as Fc fragments and intact IgG. Furthermore, Lee and Painter (145) reported that a peptide derived from the CH_2 domain (residues 253–306) also bound C1q in the fluid phase C1-binding assay.

Although activation of the C system has been viewed in the context of a predominantly nonspecific protective mechanism, there is evidence indicating that C components are involved in the regulation of specific humoral and cell-mediated immunity. This concept first arose from the observation that leukocytes possess receptors for various fragments of C (149,150). The third component of C (C3) has especially been implicated in the regulation of immune responsiveness. C3 and its fragments have been reported to (1) localize antigen in germinal centers (151,152), (2) inhibit thymus-dependent antibody responses (153–157), (3) inhibit mitogen- and antigen-induced lymphocyte proliferation (158–162), (4) induce lymphokine secretion (163–165), (5) enhance serum-induced mitogenesis, and (6) be mitogenic for B and T cells (166–168). Due to the large number of fragments and the complex nature of immunoregulation, there are many studies that are contradictory, making it difficult to interpret the role of C in immunoregulation (169,170).

B. Regulation of Specific Humoral Immune Responses

Complexes formed between antigen and antibody initiate reactions resulting in the release of biologically active mediators and tissue injury (109,171–173). Furthermore, the presence of IC locally and in circulation is used as an indicator of immunologic disease states (109,173). IC have recently been implicated as part of a complex regulatory system involved in the control of immune responses (109,171,174–176). Numerous investigators have reported that administration of passive antibody along with antigen results in suppression

of humoral immune responses (109,171,174–182). The ability of passive antibody to suppress is influenced by (1) time interval between the administration of antibody and challenge with antigen (183,184), (2) class of antibody (185–188), and (3) affinity of the antibody for immunogen (184). It was first postulated that passive antibody functions by masking specific antigenic determinants, thus inhibiting their presentation to receptors on macrophages and lymphocytes. Cerottini *et al.* (188,189) demonstrated that antibody directed to certain determinants on protein antigens inhibited the *in vivo* response to these determinants, but not to unrelated determinants on the same molecule. They also reported that $F(ab')_2$ fragments were as effective in inhibiting thymus-dependent immune responses as intact IgG antibody. Similarly, Feldmann and Diener (190) reported that $F(ab')_2$ fragments of IgG could inhibit antibody responses to thymus-independent antigens. In contrast to these findings, other investigators have reported that the Fc region of passive antibody is necessary for suppression (187,191–197). Kappler *et al.* (198) observed that passive antibody to one antigenic determinant inhibited the humoral immune response to both the specific determinant and to unrelated antigenic determinants. Furthermore, these authors (198) observed that $F(ab')_2$ fragments of antibody were unable to suppress antibody production. Chan and Sinclair (199) postulated that $F(ab')_2$ fragments of antibody suppressed immune responses by masking antigenic determinants, whereas intact antibody additionally altered immune function through a pathway involving the Fc region. Hoffmann and Kappler (200) suggested that antibody-mediated suppression of antibody synthesis occurred through two mechanisms: (1) one mechanism operates at low concentrations of antibody and requires the Fc portion of antibody, and (2) the other mechanism requires high concentrations of antibody and does not involve the Fc portion of antibody. These authors proposed that in the presence of low antibody concentrations (mechanism 1), the antibody interferes with interactions between T and B cells. This was concluded from their observations that suppression by low doses of antibody could be reversed by factors which substitute for helper T cell functions. One would predict that interference with antibody responses to thymus-independent antigens would occur through mechanism 2, i.e., only occur with high doses of antibody. Indeed, Feldmann and Diener (190) observed that inhibition of the *in vitro* antibody responses to polymerized flagellin, a thymus-independent antigen, by passive antibody was independent of the Fc region and required large amounts of antibody.

The Fc portion of antibody is also required for allotypic (201,202) and anti-idiotypic (203,204) suppression. More recently, it has been reported that guinea pig anti-idiotypic antibody could suppress or amplify the immune response in mice depending upon the class of anti-idiotypic antibody used (204). In view of the postulated role for anti-idiotypic suppression in the network theory of immunoregulation (205,206), the interaction of the Fc portion of complexed antibody may be a critical event in immunoregulation.

Suppression of humoral immune responses by passive antibody or IC could result from the cross-linking of antigen and FcR (207–214). One of these studies (211) suggests that antibody-mediated inhibition depends on the presence of FcR-bearing cells functioning as targets for the Fc region of antibody. Sidman and Unanue (208) showed that anti-Ig antibodies could inhibit LPS-induced B cell proliferation. These authors (208) suggest that inhibition occurs when the anti-Ig cross-links with the surface Ig and FcR. In addition, Ryan and coworkers (209,210) showed that IC inhibited concanavalin A-induced T cell proliferation. Further, La Via and La Via (211) reported that aggregated Ig could inhibit *in vitro* antibody responses to both T cell-dependent and -independent antigens. Ryan and Henkart (212) suggested that inhibition of immune function by interaction of IC and FcR could occur by a number of mechanisms which include (1) immobilizing receptors, (2) inhibiting binding to cell surface receptors, and (3) transmission of inhibitory signals through the binding to FcR.

IC-mediated suppression of humoral immune responses could also occur through the activation of suppressor T cells. T cells obtained from mice and humans are known to express FcR for IgG or IgM (78,80,82). Moretta *et al.* (84) observed that IC will induce T cell-mediated nonspecific suppression of pokeweed mitogen-induced stimulation of human B cells. These authors (215) further demonstrated that T cells bearing FcR for IgG are responsible for suppressing polyclonal activation of human B cells after encountering IgG containing IC. Furthermore, it was observed that preculture of IC with T cells bearing IgG FcR resulted in decreased proliferation in response to T cell mitogens (216). Taylor and Basten (217) reported that IC-induced suppressor T cells of mice regulated immune responses by activating macrophages to secrete suppressor factors. Additional support for the involvement of FcR^+ T cells in immunoregulation is that Con A-induced nonspecific suppressor cells are FcR positive (218).

IC-mediated suppression has been shown to occur through the release of soluble suppressor molecules. Activated mouse T cells have

been reported by Fridman and co-workers to release a protein, IgG-binding factor (IgG-BF), which inhibits IgM and IgG synthesis in response to thymus-dependent and -independent antigens (219–223). IgG-BF binds preferentially to IC rather than monomeric IgG. Detailed analysis of IgG-BF (223) suggests that IgG-BF and the FcR for IgG may be the same molecule. These authors (222,223) suggest that IgG-BF inhibits by binding to antigen–antibody complexes and other molecules on the B cell membrane. In addition to T cells, macrophages and B cells have been reported to secrete suppressor substances upon interaction with IC. FcR-positive B cells derived from normal mouse spleen have been shown to nonspecifically suppress antibody production by FcR negative B cells (224). This suppression was mediated by a factor released from the FcR-bearing B cells. Further work by these investigators suggest that the suppressor factor is unrelated to either IgG-BF or Ig (225). Aggregated Ig (226,227) and IC (228) have been reported to stimulate the release of metabolic products of arachidonic acid (AA) from macrophages and monocytes. AA metabolites, primarily oxidative products such as prostaglandin (PG), have been shown by numerous investigators to down regulate immune responses (229–231). These observations are of special importance in light of the reported associations of the FcR and the enzyme phospholipase A_2 (PLA_2) (232–234). One of the rate-limiting steps in PG synthesis is the activation of PLA_2, which catalyzes hydrolysis of the ester bond at the C-2 position of membrane-bound phospholipids to release AA, the precursor of PG (235).

Although emphasis has been placed on the suppression of the immune response by passive antibody, enhancement has also been reported by a number of investigators. Such enhancement has been observed with antibody responses to both soluble (236–240) and particulate (241–249) antigens and apparently results from an increase in immunogenicity of antigen complexed to antibody in antigen excess over that of free antigen. Walker and Siskind (184) demonstrated that low concentrations of high-affinity antibody to the dinitrophenyl (DNP) hapten enhanced antibody responses to this hapten. Similarly, Pincus *et al.* (240) observed that low doses of antisera to bovine serum albumin (BSA) in mice injected with DNP-BSA resulted in enhanced antibody responses to both the hapten and carrier. In addition, poor immunogens can be made strongly immunogenic by complexing with antibody. Tite *et al.* (180) showed that injection of stable, covalently bonded monomeric complexes of rabbit anti-NAP (4-azido-2-nitrophenyl) antibodies and NAP-bovine pancreatic ribonuclease

(RNase) into mice primed these animals to RNase, whereas injection of RNase alone had no such effect.

As observed *in vitro*, regulation of immune reactivity by antibody *in vivo* is also dependent upon the ratio of antibody to antigen. Thus, complexes of different ratios apparently have different biological properties, some resulting in enhancement while others result in suppression. Experiments on immune regulation by antibody led Bystryn *et al.* (250) to propose that antibody production to both persisting and readily catabolized antigen is controlled via a dynamic equilibrium among circulating antibody, antigen, and antigen–antibody complexes. Thus, depending on a variety of conditions, complexes of changing ratios could exist at various times during *in vivo* immune responses leading to a dynamic regulation of the responses.

Enhancement as well as suppression by passive antibody or IC has been shown to be dependent upon the class or subclass of antibody (185,243,245,246,248,249,251). Murgita and Vas (185) observed suppression of the immune response in mice to SRBC at all concentrations of IgG_1 antibody, while suppression with IgG_2 antibody was observed only with high concentrations and enhancement was found with low concentrations. In general, IgM antibodies have been shown to enhance and IgG antibodies to suppress the humoral immune response to antigen (245–249). Mechanisms put forward to account for enhancement of humoral immune responses by IC include (1) increased localization of antigen in lymphoid follicles when antigen is complexed with antibody (252), (2) improved handling of antigen by macrophages (253), (3) more efficient presentation of antigen for cellular interaction (254), and (4) passive antibody may carry a cross-reactive idiotype (255) which could potentially amplify the antibody response by activating idiotype specific helper T cells (256), thus triggering the release of biologically active cytokines. Chou *et al.* (257) observed that IC bound to human erythrocytes trigger the release of interleukin-1 (IL-1) from human monocytes.

In addition to modulating specific immune responses, IC and aggregated Ig have been shown to directly activate lymphocytes *in vitro* in an antigen-nonspecific manner. Bloch-Shtacher *et al.* (258) observed that exposure of human lymphocytes to IC resulted in morphological changes in the cells and an increased uptake of [^{14}C]thymidine into DNA. Similarly, Moller (259) reported that preformed antigen–antibody complexes were able to induce DNA synthesis in normal human lymphocytes cultured *in vitro*, whereas antibody or antigen alone had a negligible effect. More recently, rheumatoid factor-containing im-

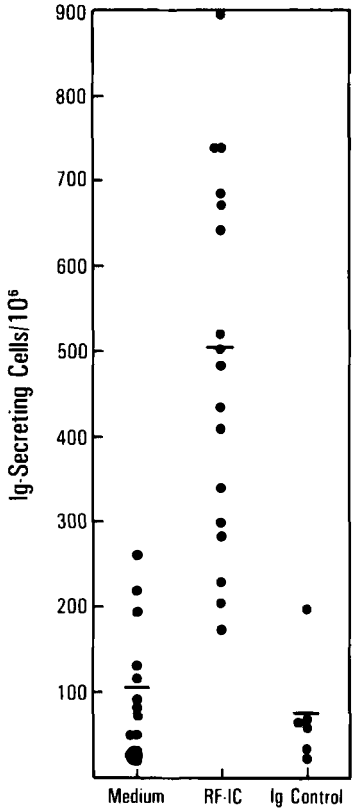

FIG. 4. Polyclonal antibody production induced by rheumatoid factor-associated immune complexes (RF-IC) derived from plasma from seropositive patients. Ig-secreting cells were measured on day 6 of culture by the protein A plaque assay. Reprinted from Hobbs *et al.* (1985). *J. Immunol.* **134**, 223.

mune complexes (RFIC) isolated from the plasma of seropositive patients were shown to increase the number of Ig-secreting cells in normal human lymphocyte cultures (260) (Fig. 4). Aggregated Ig and IC have also been reported to nonspecifically activate human (261), rabbit (262), and mouse (263–265) lymphocytes. Mouse splenic B cells are stimulated to proliferate and secrete polyclonal Ig in the presence of heat-aggregated human γ-globulin (AHGG) (Table I) (263,264). The method of aggregation appears to be critical for the generation of stimulatory HGG preparations. Weigle and Berman (266) reported that mouse B cells could be activated by soluble IC. The proliferative response was found to be dependent upon the antibody–antigen ratio in the complexes. These results were later ex-

TABLE I[a]
AGGREGATED HUMAN γ-GLOBULIN-INDUCED ACTIVATION OF MOUSE LYMPHOCYTES

HGG treatment[b]	μg/ml	[3H]TdR uptake[c] (cpm ± SD)	Direct anti-TNP PFC/10^6 cells[c] (± SD)
—	—	2,544 ± 685	40 ± 17
None	3	2,343 ± 796	ND[d]
None	30	1,888 ± 529	44 ± 4
None	300	2,039 ± 115	53 ± 6
Heat aggregated	3	2,932 ± 409	ND
Heat aggregated	30	7,959 ± 144	ND
Heat aggregated	300	26,599 ± 410	244 ± 17

[a] Reprinted from Morgan and Weigle (1980). *J. Immunol.* **125**, 226.
[b] Human γ-globulin was heated at 63°C for 30 minutes.
[c] Proliferation and polyclonal antibody production measured on day 3 of culture.
[d] ND, Not done.

tended to show that homologous IC (again depending upon the ratio) could activate mouse B cells to proliferate and secrete polyclonal Ig (Fig. 5) (265). Because antigen and antibody were added independently at culture initiation, the exact nature of the complex was unknown, but optimal lymphocyte activation occurred in antigen excess. More recently, Saito-Taki and Nakano (227) reported that aggregated murine IgG_1 and IgG_{2a} myeloma proteins enhanced LPS-induced polyclonal Ig secretion.

C. REGULATION OF CELL-MEDIATED IMMUNE RESPONSES

Although the majority of available data on regulation of the immune response by IC is involved with humoral immunity, cell-mediated immunity can also be modulated by IC (267–271). MacKaness and coworkers (268) reported that immunization procedures which favor the induction of humoral immunity interfere with the generation of delayed type hypersensitivity (DTH). Sinclair *et al.* (272) have demonstrated that sensitization of mouse T cells with allogeneic cells is suppressed by antibody specific for the sensitizing cells. Recently, Schalke *et al.* (273), studying IC-mediated regulation of specific T cell clones, found that the addition of small amounts of monoclonal anti-acetylcholine receptor (AChR) with suboptimal concentrations of AChR to specific T cell clones produced an enhanced proliferation of the T cell clones. Enhanced activation was found to be dependent on the isotype of monoclonal antibody used. Similar results have been

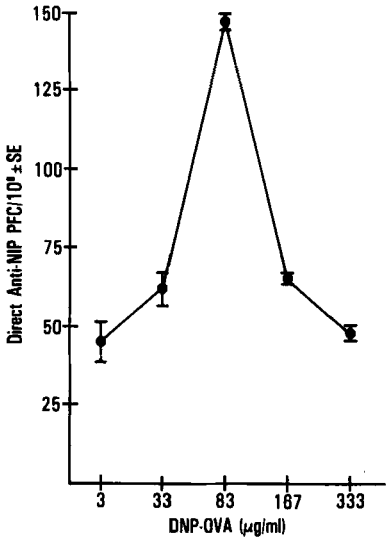

FIG. 5. Polyclonal antibody production by mouse spleen cells upon stimulation with monoclonal mouse IgM anti-DNP and DNP-OVA complexes. The antibody concentration was held constant at 250 μg/ml, and increasing amounts of DNP–OVA were added to culture. Reprinted from Morgan and Weigle (1983). *J. Immunol.* 130, 1066.

reported by Celis and Chang (274), who observed that antibodies to hepatitis B surface antigen potentiate the proliferative response of human T cell clones to the same antigen.

One of the best documented associations of FcR bearing cells with an immunological event is that observed with antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC has been directly or indirectly associated with such immune phenomena as tumor allograft rejection. The ADCC effector (K) cell has been reported to be FcR positive and surface Ig negative (275,276). IC prepared in antigen excess inhibited ADCC, whereas IC prepared in antibody excess enhanced ADCC (277,278,279). Aggregated preparations of IgG also suppressed ADCC (280,281). Spiegelberg *et al.* (75) reported that aggregated IgG_1 and IgG_3 myeloma proteins markedly inhibited ADCC, while aggregated IgG_2 and IgG_4 were much less effective. Although aggregated preparations of IgG_2 and IgG_4 inhibited ADCC, small amounts of unaggregated IgG_2 and IgG_4 enhanced ADCC. In addition to K cells, natural killer (NK) cells also bear FcR (282–284). IC (285–286) and aggregated Ig (287) have uniformly been shown to suppress NK activity. Merrill *et al.* (284) reported that IgG-containing IC are more suppressive than IgM-containing complexes. These authors pos-

tulated that the IgG-containing complexes block the lytic event in NK activity by binding to FcR.

D. Nonspecific Lymphocyte Activation by Fc Fragments of Ig

The Fc portion of the Ig molecule is responsible for a wide variety of biological functions that the Ig molecule and IC possess. Recently, it has been shown that Fc fragments of Ig possess a number of important immunoregulatory functions shown to be associated with IC and aggregated Ig (226,263–266,288–292). Berman and Weigle (263) observed that Fc fragments derived from enzymatic cleavage of the Ig molecule induce murine spleen cells to proliferate. The proliferative response was specific for the Fc fragments in that proliferation could not be induced by intact monomeric IgG, Fab, or $F(ab')_2$ fragments. This mitogenic activity was originally described with Fc fragments prepared from a commercial source of HGG. It has been shown that Fc fragments prepared from human IgG_1, IgG_2, IgG_3, IgG_4, IgD, IgM, and IgA myeloma proteins, as well as Fc fragments prepared from goat and mouse IgG, are effective in inducing mouse spleen cell proliferation (266,293). This is an interesting observation in light of the findings of Spiegelberg *et al.* (75), where Fc fragments derived from some human myeloma proteins enhanced ADCC *in vitro* and that these Fc fragments could also induce lymphocyte-mediated target cell lysis in the absence of added antibodies. Addition of Fc fragments from human IgG_2 and IgG_4 induced a dose-dependent cytotoxicity to chicken erythrocytes (CRBC) in the absence of anti-CRBC antibodies. In contrast to these results, aggregated IgG_2 and IgG_4 inhibited ADCC. The observations of Spiegelberg *et al.* (75) led to the hypothesis that two active sites are present in the Fc region of antibody, one having "triggering" action and the other having high-affinity binding activity to FcR. This hypothesis is also supported by the findings of Berman and Weigle (263), which showed that Fc fragments derived from papain cleavage of IgG induced murine B cells to proliferate, whereas intact IgG was unable to activate B cells.

The cell type induced to proliferate in the presence of Fc fragments is a B cell (263,294). B cells derived from athymic nude mice (Table II) or from spleen cells treated with anti-thymocyte serum + C proliferated in the presence of Fc fragments to the same level as intact spleen cells. In contrast, nylon wool-purified T cells did not proliferate in response to Fc fragments. Subsequent analysis revealed that the cells responding to Fc fragments were Ig^+, FcR^+, Ia^+, and CR^+ B cells (263,294).

TABLE II[a]
Fc Fragment-Induced Proliferation of Nude Mouse Spleen Cells

Spleen cell source[b]	Stimulator	μg/ml	[^{3}H]TdR uptake[c] (cpm ± SD)
nu/nu	Fc fragments	200	33,647 ± 1,825
nu/+	Fc fragments	200	30,064 ± 937
nu/nu	LPS	40	146,157 ± 8,300
nu/+	LPS	40	177,241 ± 2,005

[a] Reprinted from Morgan and Weigle (1980). *J. Immunol.* **124,** 1330.
[b] Spleen cells were from C57BL/6 athymic (*nu/nu*) or C57BL/6 athymic littermates (*nu/+*).
[c] Proliferation was measured on days 2–3 of culture. Background cpm did not exceed 2500 and was subtracted from the response.

The proliferative response of mouse splenic B cells to Fc fragments requires a population of adherent accessory cells (288). Filtration of mouse spleen cells through columns of Sephadex G-10 abrogated their ability to respond to Fc fragments, but not their ability to respond to LPS (Table III). The splenic adherent cells appear to be macrophages because they (1) are depleted by Sephadex G-10 filtration, (2) adhere to plastic, (3) phagocytize carbonyl iron, (4) are radiation resistant, (5) are esterase positive, and (6) are resistant to antithymocyte serum + C (288,295). The response of Sephadex G-10-filtered cells can be restored by the addition of splenic adherent macrophages (Table IV). The role of the macrophage apparently is to cleave the Fc

TABLE III
Requirement for Macrophages in the Fc Fragment-Induced Proliferative Response

Spleen cell treatment	Fc fragments[a]	LPS[b]	[^{3}H]TdR uptake[c] (cpm ± SD)
No treatment	−	−	5,137 ± 1,904
No treatment	+	−	59,300 ± 1,541
No treatment	−	+	76,592 ± 3,666
G-10 filtered	−	−	5,675 ± 714
G-10 filtered	−	−	7,612 ± 4,398
G-10 filtered	−	+	82,159 ± 2,262

[a] 250 μg/ml.
[b] 50 μg/ml.
[c] The proliferative response was measured on days 2–3 of culture.

TABLE IV[a]
RESTORATION OF THE Fc FRAGMENT-INDUCED PROLIFERATIVE RESPONSE BY SPLENIC ADHERENT MACROPHAGES

Spleen cell treatment[b]	Adherent cell treatment	[^{3}H]TdR uptake[c]
Untreated	—	30,265 ± 1,524
G-10 filtered	—	1,978 ± 407
G-10 filtered	Irr, Anti T + C	29,795 ± 1,798

[a] Reprinted from Morgan and Weigle (1979). *J. Exp. Med.* **150,** 257.
[b] Responder cells were cultured with 250 μg Fc/ml.
[c] The response was measured on days 2–3 of culture. Background cpm did not exceed 3000 and was subtracted from the experimental response.

fragments into smaller fragments which activate B cells (64,295). Addition of Fc fragments to macrophage monolayers results in the generation of culture supernatants that induce macrophage-depleted spleen cells to proliferate. It is important to note that supernatants derived from untreated macrophage monolayers do not induce proliferation. It appears that the active substance in the Fc-macrophage supernatants is derived from the Fc fragment, since filtration of the Fc-macrophage supernatant through an anti-Fc fragment affinity column renders the effluent incapable of inducing B cell proliferation (Table V). Acid elution of the anti-Fc fragment column results in the recovery of material which induces B cell proliferation. In contrast to these results, passage of the Fc–macrophage supernatant over an anti-bovine serum

TABLE V[a]
RECOVERY OF MITOGENIC ACTIVITY FROM Fc-MACROPHAGE SUPERNATANT BY ANTI-Fc FRAGMENT AFFINITY CHROMATOGRAPHY[b]

Fc–macrophage supernatant	Supernatant treatment[c]	[^{3}H]TdR uptake[d] (cpm ± SD)
+	None	44,510 ± 4,578
+	Anti-Fc column effluent	4,714 ± 1,388
+	Anti-Fc column eluate	30,214 ± 2,573

[a] Reprinted from Morgan and Weigle (1980). *J. Exp. Med.* **151,** 1.
[b] Sephadex G-10-filtered spleen cells were stimulated with supernatant derived from a 30-minute coculture of adherent macrophages and Fc fragments.
[c] Supernatant was filtered twice (2×) through an anti-Fc fragment column and the column effluent and eluate assayed for mitogenic activity.
[d] The response was measured on days 2–3 of culture. Background cpm did not exceed 3000 cpm and was subtracted from the experimental response.

TABLE VI[a]
REMOVAL OF MITOGENIC ACTIVITY FROM FC–MACROPHAGE SUPERNATANT MATERIALS[b]

Fc–macrophage supernatant	Supernatant treatment[c]	[^{3}H]TdR uptake[d] (cpm ± SD)
+	None	39,813 ± 1,151
+	Anti-Fc column effluent (1×)	12,393 ± 767
+	Anti-Fc column effluent (2×)	3,229 ± 1,094
+	Anti-BSA column effluent (1×)	37,166 ± 1,524
+	Anti-BSA column effluent (2×)	44,119 ± 5,192

[a] Reprinted from Morgan and Weigle (1980). *J. Exp. Med.* **151**, 1.

[b] Sephadex G-10 filtered spleen cells were stimulated with supernatant derived from a 30-minute coculture of adherent macrophages and Fc fragments.

[c] Supernatant was filtered once (1×) or twice (2×) through either an anti-Fc fragment or anti-BSA affinity column prior to assaying for mitogenic activity.

[d] The response was measured on days 2–3 of culture. Background cpm did not exceed 3000 and was subtracted from the experimental response.

albumin column does not deplete biological activity from the supernatant (Table VI). In addition to mouse splenic macrophages, the $P388D_1$ macrophage-like cell line has been shown to process Fc fragments but not intact monomeric IgG_1 into subfragments capable of inducing mouse B lymphocytes to proliferate in the absence of macrophages. Processing of Fc fragments by murine macrophages (289) or the $P388D_1$ macrophage-like cell line (64) is a rapid event, with proliferation-inducing components detectable after 10 minutes of incuba-

TABLE VII
FC FRAGMENT-MEDIATED POLYCLONAL IG SECRETION[a]

Stimulator	Direct anti-TNP PFC/10^6 cells	Total Ig (μg/ml)
	Experiment 1	**Experiment 2**
None	10	<0.3
Fc fragments	190	29.1
LPS	470	101.7

[a] Spleen cells were stimulated with Fc fragments or LPS and the polyclonal antibody response measured on day 3 by hemolytic plaque formation or day 5 for supernatant antibody by ELISA.

tion. Although the enzyme(s) responsible for subfragment generation are unknown, a number of macrophage-derived proteases have been characterized. One possibility is elastase, a metalloproteinase secreted by activated macrophages (296). This enzyme has been shown to degrade both murine and human IgG as well as Fc fragments derived from murine IgG_{2a}; and, in the latter case, 14,000 M_r subfragments were produced (297), which may correspond to the mitogenic subfragments described by this laboratory (289). Macrophages are also known to produce plasminogen activator (298) which, in the presence of plasminogen, may form plasmin, a known generator of proliferation-inducing Fc subfragments (299).

In addition to inducing B cell proliferation, Fc fragments stimulate mouse (263,289,300) and human (292,301) B cells to secrete polyclonal Ig (Table VII). In contrast to the proliferative response, Fc fragment-induced polyclonal Ig secretion requires the presence of both macrophages and T cells (289,292,299,300). The addition of Fc fragments to cultures of spleen cells from athymic or conventional T cell-depleted mice results in a proliferative response but no polyclonal Ig secretion, although the same cell populations proliferate and secrete polyclonal Ig in the presence of LPS. Macrophages are also required for Fc fragment-induced polyclonal Ig production. Depletion of macrophages abolishes the ability of the remaining cells to make polyclonal Ig in response to Fc fragments, but does not diminish their ability to respond to LPS (289). The macrophage requirement can be bypassed by employing macrophage-processed Fc subfragments as the stimulator (289,299).

B cells derived from human peripheral blood mononuclear (PBM) cell populations are also induced to secrete polyclonal Ig upon stimulation with Fc fragments derived from a human IgG_1 myeloma protein (292,302) (Fig. 6). Fab fragments and intact monomeric IgG_1 do not exhibit these activities. Fc fragment-induced polyclonal Ig production requires the presence of adherent monocytes. As with mouse macrophages, human monocytes process Fc fragments into biologically active Fc subfragments capable of activating human lymphocytes. T cells are also required for the secreting of polyclonal Ig in the presence of Fc fragments (292,302) (Fig. 7). Early experiments indicated that intact PBM populations were inconsistent in their ability to secrete Ig when stimulated with Fc fragments. It was observed that irradiation of T cells (2000 R) prior to recombination with B cells + monocytes overcame this inconsistency. These results are in agreement with published observations (303,304), which showed that T cell irradiation increased polyclonal Ig production in human PBM cul-

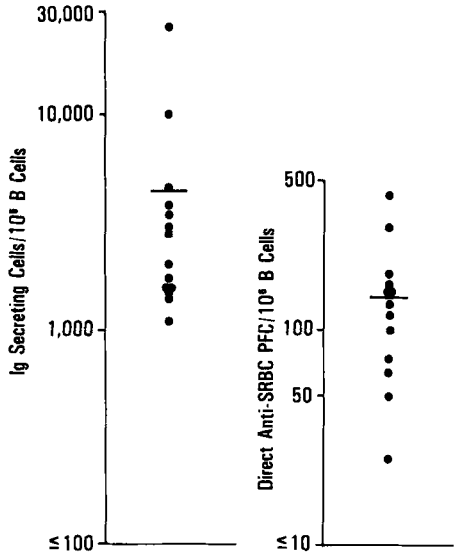

FIG. 6. Comparison of polyclonal antibody production of human B cells induced by Fc fragments (3 μg/ml). Reprinted from Morgan and Weigle (1980). *J. Exp. Med.* **154**, 778.

tures to pokeweed mitogen. It was suggested that irradiation preferentially depletes nonspecific suppressor T cell populations (303,304).

The T cells signal required for Fc fragment-induced polyclonal Ig secretion can be delivered by a soluble B cell-activating factor generated by T cells exposed to Fc fragments (305–307). Addition of Fc

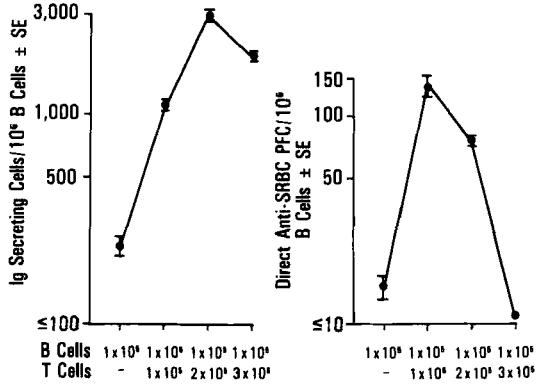

FIG. 7. Optimal B cell : T cell ratio for the Fc fragment-induced polyclonal antibody response. Fc fragments were used at 3 μg/ml. Reprinted from Morgan and Weigle (1980). *J. Exp. Med.* **154**, 778.

TABLE VIII[a]
STIMULATION OF BCAF PRODUCTION BY Fc FRAGMENTS

Fc fragments (μg/ml)[b]	Direct anti-TNP PFC/10^6 cells (± SE)
—	25 ± 7
50	153 ± 20
100	218 ± 3
150	83 ± 10

[a] Reprinted from Thoman *et al.* (1980). *J. Supramol. Struct.* **13**, 479.

[b] Spleen cells (5×10^4) were cultured for 24 hours with Fc fragments. Culture supernatants were tested for BCAF activity in the Fc fragment-induced polyclonal antibody response in T cell-depleted spleen cell cultures.

fragments to intact spleen cells or T cell-enriched (B cell-depleted) populations results in the production of a soluble factor. SP-BCAF-thm (BCAF) [formerly Fc-TRF (305–307)], which has the capacity to replace T cells in Fc fragment-induced polyclonal Ig secretion (Table VIII). This factor was also found in supernatants derived from Con A-stimulated spleen cell cultures (306). Fc fragments stimulate T cells of the Lyt-1^+2^- subset to release BCAF, and macrophages are necessary for factor production although this requirement can be bypassed by using macrophage-processed Fc subfragments as the stimulator. That the active entity is a T cell product and not modified Fc fragments is derived from the observation that BCAF is not depleted by filtration through an anti-Fc fragment affinity column. Preliminary chemical characterization of the factor indicates that biological activity can be separated into two major size classes of 55,000–60,000 and 35,000–40,000 M_r by Sephadex G-100 filtration (Fig. 8). When the factor was produced in serum-free medium, a third, 100,000 M_r component was found, which is most likely aggregated material (307). Although the relative amounts of activity present in each peak varies somewhat between preparations, the differences do not appear to be significant. Two isoelectric point species have also been identified, focusing at pH 3.3 and 4.2 (Fig. 9). Multiple analyses reveal that the isoelectric point of the more basic species may vary between 3.9 and 4.3. To date it has not been determined whether or not one size class represents one isoelectric species.

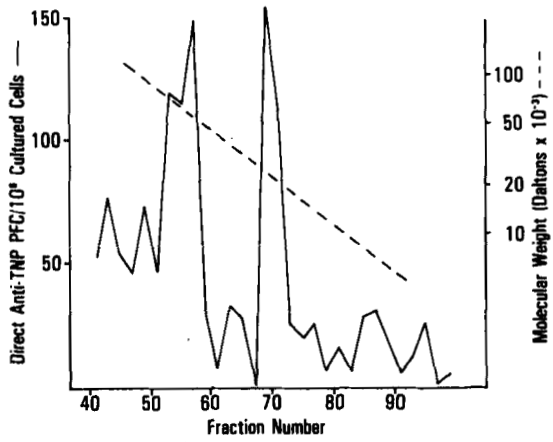

FIG. 8. Fc fragment-induced BCAF was subjected to Sephadex G-100 chromatographic separation. Murine spleen cells were stimulated with 50 μg Fc fragments/ml for 24 hours. Twenty-five milliliters of concentrated supernatant was applied to the column and eluted with 0.9% saline. Three-milliliter fractions were collected and assayed for BCAF activity by their ability to replace T cells in the Fc fragment-induced polyclonal antibody production model. Reprinted from Thoman and Weigle (1982). *J. Immunol.* **128**, 590.

The observations that both macrophages and T cells are independently required for Fc fragment-induced polyclonal Ig production implies that B cell differentiation in this system occurs through the delivery of at least two distinct signals (Fig. 10). One signal is associated

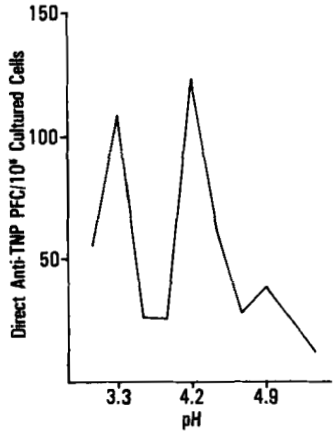

FIG. 9. Isoelectric focusing (IEF) pattern of BCAF. Twenty-four milliliters of culture fluid from FC fragment-stimulated spleen cells was subjected to IEF. Reprinted from Thoman and Weigle (1982). *J. Immunol.* **128**, 590.

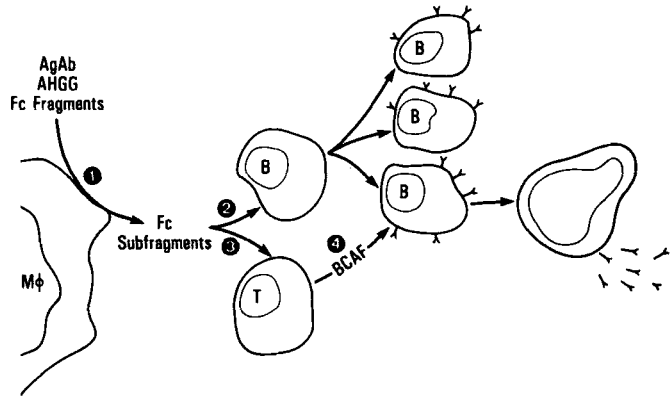

FIG. 10. Two signal models for Fc fragment-induced polyclonal Ig production.

with proliferation and is generated by the interaction of the mitogenic Fc subfragment with B cells. A second signal is apparently delivered by BCAF generated by T cells following their activation by Fc subfragments and the latter signal results in the differentiation of B cells to Ig-secreting cells. An alternate hypothesis to the two signal model is that one subpopulation of B cells is stimulated to proliferate, while another is stimulated to undergo differentiation to Ig-secreting cells. This second hypothesis is unlikely because the Ig-secreting cells are contained in the proliferating population as evidenced by "hot pulse" experiments (289). The presence of highly labeled thymidine for the first 2 days of culture reduced the Ig response to Fc fragments (Table IX). In contrast, when the thymidine was present for only the last 24 hours of culture, there was no reduction in the polyclonal Ig secretion. These results are interpreted to mean that the necessary proliferative event occurs during the first 48 hours of culture and precedes differentiation to Ig-secreting cells. Similar models for mouse (308) and human (309) B cell activation by anti-Ig have recently been described. Activation of mouse B cells by soluble anti-Ig or anti-Ig-coupled polyacrylamide beads results in a pronounced proliferative response but no polyclonal Ig secretion. However, in the presence of supernatants derived from Con A-activated spleen cells, B cells respond to anti-Ig-coated beads by proliferating and secreting polyclonal Ig (308). Similarly, Yoshizaki *et al.* (309) observed that stimulation of leukemic B cells (B-CLL) with anti-idiotypic antibody and cell-free supernatants from phytohemagglutinin (PHA)-stimulated T cells induced monoclonal IgM secretion. Neither anti-idiotypic antibody nor PHA supernatants alone induced any IgM secretion, confirming the previous

TABLE IX[a]
EFFECT OF HOT PULSE OF [^{3}H]THYMIDINE ON Fc FRAGMENT-INDUCED Ig SECRETION[b]

Duration of hot [^{3}H]TdR pulse[c]		Direct anti-TNP PFC/10^6 cells (± SD)
Day	**Hour**	
—	—	118 ± 11
0	0–72	5 ± 3
1	24–72	11 ± 8
2	48–72	91 ± 14

[a] Reprinted from Morgan and Weigle (1980). *J. Immunol.* **124,** 1330.

[b] 200 μg Fc/ml were added at culture initiation.

[c] 20 μCi [^{3}H]TdR were added at 0, 24, and 48 hours and the cultures assayed for PFC at 72 hours. Background cpm did not exceed 10 PFC and was subtracted from the experimental response.

results that at least two sets of signals are required for B cell activation into Ig-secreting cells.

Fc fragment-induced BCAF acts on activated B cells, stimulating their differentiation to Ig-secreting cells. However, no T cell growth-promoting or costimulator activity can be demonstrated in the BCAF-containing supernatants (307) (Fig. 11), indicating that BCAF is distinct from interleukin-2 (IL-2). Fc fragment-induced BCAF is also distinct from currently known B cell growth factors (BCGF) (310–312). The presence of two distinct kinds of BCGF has been demonstrated by the use of short-term B cell costimulator assays (313) and the murine leukemic B cell line BCL_1 (314,315). BCGF-I synergizes with anti-Ig in the proliferation of normal mouse splenic B cells and fails to directly stimulate the BCL_1 cell line to proliferate (309,310,313,316,317), while in contrast, BCGF-II fails to synergize with anti-Ig to induce proliferation of normal B cells but induces proliferation of Dextran sulfate-stimulated B cells as well as directly activating the BCL_1 line (313,315,317). Fc fragment-induced BCAF is unable to synergize with anti-Ig in the BCGF-I assay or synergize with Dextran sulfate in the BCGF-II assay (M. L. Thoman, unpublished observations). Moreover, BCAF does not induce BCL_1 cells to proliferate.

Fc fragment-induced BCAF more closely resembles B cell differentiation factors (BCDF), which do not have growth activity and are involved in the final differentiation of B cells to Ig-secreting cells.

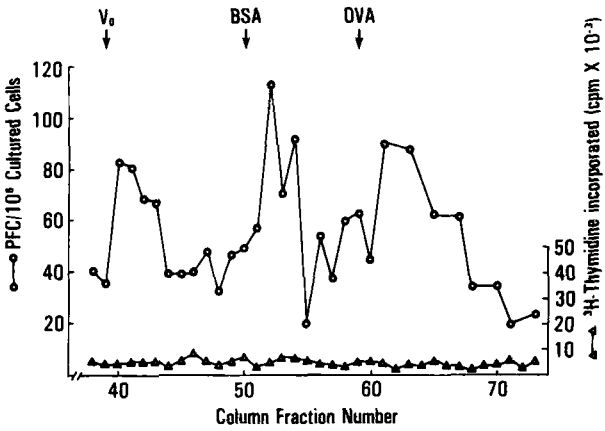

FIG. 11. Absence of IL-2 activity in Fc fragment BCAF preparations. Supernatant from spleen cells stimulated with 50 μg Fc fragments/ml was subjected to Sephadex G-100 chromatography and fractions assessed from BCAF activity (O—O) and IL-2 activity (Δ—Δ). Reprinted from Thoman and Weigle (1982). *J. Immunol.* **128**, 590.

Supernatants from mitogen-stimulated T cells and cloned T cell lines have been shown to synergize with mitogens in inducing normal B cells to secrete Ig and directly activate B tumor cells to secrete Ig (318–321). Kishimoto and Ishizaka (322) reported that anti-Ig activated B cells into a state responsive to T cell factors. Similar results were obtained by Parker *et al.* (308), who demonstrated that insolubilized anti-Ig and Con A supernatants could synergize to induce more spleen cells to secrete Ig. In addition to anti-Ig-induced stimulation of normal B cells, human (318,320) and mouse cell lines have been employed to study BCDF activity. Muraguchi *et al.* (323) established a human B lymphoblastoid line (CESS) that was responsive to a BCDF. IgG secretion was induced in CESS cells within 48 hours by the addition of supernatants from PHA-stimulated human T cells. Furthermore, BCDF did not affect the proliferation of CESS cells, and the blocking of cell proliferation by hydroxyurea did not inhibit the increase in IgG-secreting cells. BCDF released from PHA-stimulated human T cells was shown to be 20,000 M_r with a p*I* of 5.5–5.7 (309,324). In addition to a 20,000 M_r BCDF, Teranishi *et al.* (324) reported the existence of a 35,000 M_r species. More recently, Butler *et al.* (325) reported a human T cell hybridoma-derived BCDF which was 30,000–35,000 M_r with a p*I* of 5.9. In addition to human BCDF, BCDF activity has also been generated in the mouse model. The BCL_1 cell can be induced to secrete IgM upon stimulation with supernatants derived from T cell sources (326). The BCDF was shown to be

30,000–60,000 M_r in size. Sidman *et al.* (327) have also measured BCDF activity using B cell tumor lines. These authors observed that a cell line resembling normal resting B cells (WEHI-279) could be stimulated by BCDF to secrete Ig. Moreover, 707/3 cells which resemble pre-B cells are induced to initiate L chain synthesis and display detectable surface Ig within 24 hours of culture with BCDF (328). The BCDF was characterized as being 50,000–55,000 M_r by gel filtration and 16,000 M_r by SDS–PAGE with a p*I* of 5–6. Thus, Fc–BCAF resembles BCDF described by numerous investigators. Fc-BCAF appears to be involved in the final differentiation of B cells to Ig-secreting cells.

E. Enhancement of Specific *in Vitro* Humoral Immune Responses by the Fc Portion of Ig

In addition to the ability of Fc fragments to induce B cells to proliferate and secrete polyclonal Ig, Fc fragments derived from homologous and heterologous sources can regulate *in vitro* humoral immune responses. Fc fragments derived from human and mouse Ig potentiate *in vitro* specific antibody responses under the appropriate experimental conditions (290,329–332) (Table X). Fc fragment-induced enhancement of mouse *in vitro* specific antibody responses is dependent upon the dose of antigen added in culture. Addition of Fc fragments with SRBC to *in vitro* cultures resulted in a pronounced

TABLE X[a]
Fc Fragment-Induced Enhancement of the *in Vitro* Anti-SRBC Response by Mouse Spleen Cells

Stimulator[b]	SRBC[c]	Direct anti-SRBC PFC/10^6 cells[d] (± SD)
—	−	<25
—	+	106 ± 20
Fc fragments	−	80 ± 3
Fc fragments	+	858 ± 98
Fab fragments	−	<25
Fab fragments	+	141 ± 43
IgG_1	−	<25
IgG_1	+	193 ± 58

[a] Reprinted from Morgan *et al.* (1980). *J. Exp. Med.* **152,** 113.
[b] 100 μg/ml.
[c] 1×10^4 SRBC/culture.
[d] The response was measured on day 4 of culture.

enhancement of the IgM anti-SRBC response (Fig. 12) (290,329). The most pronounced increase in the anti-SRBC response occurs when suboptimal amounts of antigen are employed. The augmentation decreases as the concentration of antigen increases, and when the optimal amount of antigen is reached, Fc fragments fail to significantly affect the antibody response. The antigen dose dependency is not limited to the anti-SRBC response, since Fc fragments are also capable of enhancing the *in vitro* response to suboptimal stimulatory doses of TNP-keyhole limpet hemocyanin (E. L. Morgan and S. M. Walker, unpublished observation). That antigen concentration apparently plays a critical role in the adjuvanticity of various agents has been suggested in the literature. Sjoberg *et al.* (333) observed that the adjuvanticity of LPS for the primary *in vitro* antibody response to SRBC occurred only when low numbers of SRBC were added to culture. Moreover, Specter *et al.* (334) found that the synthetic product *N*-acetylmuramyl-L-alanyl-*O*-isoglutamine (muramyl dipeptide) enhanced the immune response of normal lymphocytes most dramatically when suboptimal numbers of SRBC were used.

Fc fragments derived from a human IgG_1 myeloma protein were

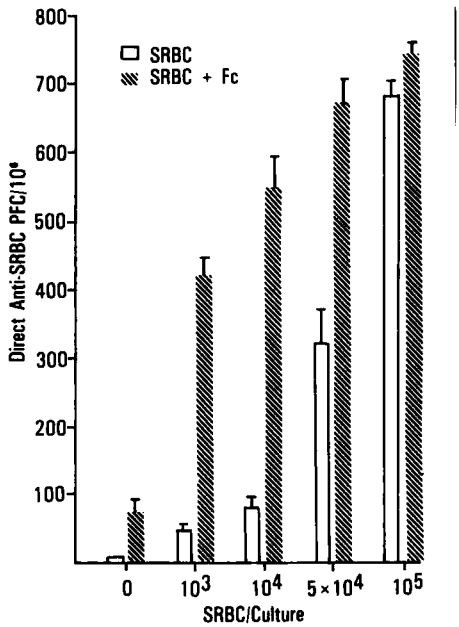

FIG. 12. Enhancement of the *in vitro* IgM anti-SRBC response. Increasing numbers of SRBC were added with 100 μg/ml Fc (shaded) or alone (open) to *in vitro* spleen cell cultures. Reprinted from Morgan *et al.* (1980). *J. Exp. Med.* **152**, 113.

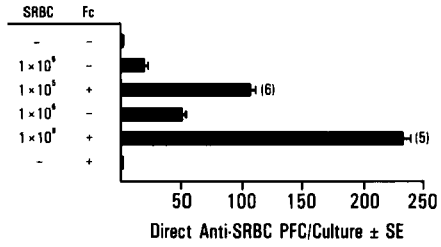

FIG. 13. Enhancement of the human primary *in vitro* anti-SRBC response. One microgram Fc fragments/ml was added to human PBM cultures along with SRBC and the anti-SRBC response measured on day 11 of culture. Reprinted from Morgan and Weigle (1983). *Clin. Exp. Immunol.* **53**, 505.

also found to be a potent adjuvant for *in vitro* human antibody responses. Addition of Fc fragments with SRBC to *in vitro* cultures of normal human PBM results in a significant increase in the number of IgM anti-SRBC plaque-forming cells (Fig. 13). Enhancement of the human *in vitro* immune response is a result of interaction of the PBM with Fc fragments and like the mouse model not a nonspecific activation due to the addition of extraneous protein to the culture environment. Addition of intact IgG_1 or Fab fragments derived from papain digestion of IgG_1 to PBM cultures did not generate the heightened anti-SRBC responses achieved when Fc fragments were used.

Augmentation of *in vitro* humoral immune responses involves the participation of T cells (329,335). To determine the role of T cells in the Fc fragment-induced enhancement of the anti-SRBC response, advantage was taken of the ability of lymphokine-containing supernatants to substitute for T cells in the anti-SRBC response (336,337). Fc fragments were unable to augment the *in vitro* anti-SRBC response when lymphokine-containing supernatant was substituted for T cells (Table XI) (329,335), suggesting a role for T cells in Fc fragment-induced enhancement of *in vitro* specific antibody responses. Further support for the participation of T cells in the Fc fragment-induced adjuvant effect comes from the findings that Fc fragments are unable to enhance antibody responses to type 1 or type 2 thymus-independent antigen (329).

It is not surprising that T cells are involved in Fc fragment-induced enhancement of *in vitro* antibody responses since T cells have been shown to play a major role in the enhancement of *in vivo* and *in vitro* immune responses by other adjuvants (338–341). In addition to Fc fragments, a major site of LPS adjuvant action in antibody responses appears to be the helper T cell (339,341). McGhee *et al.* (341), using

TABLE XI
INABILITY OF Fc FRAGMENTS TO ENHANCE THE *in Vitro* ANTI-SRBC RESPONSE WHEN LYMPHOKINE-CONTAINING SUPERNATANT IS SUBSTITUTED FOR T CELLS[a]

Lymphokine supernatant[b]	Fc fragments[c]	SRBC[d]	Direct anti-SRBC PFC/10^6 cells (± SD)
–	–	+	15 ± 3
+	–	+	342 ± 57
–	+	+	29 ± 11
+	+	+	319 ± 36

[a] T cell-depleted spleen cells were cultured with SRBC ± lymphokine supernatant and Fc fragments and the response measured on day 4 of culture.
[b] 25 μl G-100-purified Con A supernatant.
[c] 250 μg/ml.
[d] 1×10^5 SRBC/culture.

LPS responder and nonresponder strains of mice, observed that both T cells and macrophages were required for LPS-induced adjuvanticity. That the adjuvant effect of LPS can have both T cell-dependent and -independent modes of action has recently been shown. In a system employing TNP-SRBC and TNP-soluble protein antigens, it was found that in the case of TNP-SRBC, the LPS adjuvant action was T cell independent, whereas carrier-specific T cells were required for enhancement of the response to TNP-modified soluble protein antigens (342). The adjuvant properties of Fc fragments are thus different from those of LPS, in that Fc fragment action has a mandatory requirement for T cells, whereas LPS does not.

The Fc fragment-induced potentiation of the immune response could occur by several mechanisms, i.e., (1) Fc fragments may mimic the signal normally provided by helper T cells, (2) Fc fragments may affect the B cells in a manner such that the threshold for activation would be reduced resulting in a more effective use of T cell help, or (3) Fc fragments may induce an expansion in the number of helper T cells. It is possible to rule out the first of these mechanisms, because T cell-depleted cultures that received Fc fragments + antigen failed to respond (329). Similarly, the last mechanism can be partially ruled out because Fc fragments do not induce T cells to proliferate (263). Fc fragments appear to potentiate *in vitro* humoral immune responses at least in part through the production of T cell-derived BCAF (329,330,335,343). As mentioned above, the substitution of lymphokine-containing supernatants for T cells did not permit Fc fragment-

induced augmentation of the anti-SRBC response, indicating that for the adjuvant effect Fc fragments directly stimulate T cells. Furthermore, although lymphokine-containing supernatants restore the anti-SRBC response of T cell-depleted spleen cells, Fc fragment-induced BCAF added along with lymphokine-containing supernatants to T cell-depleted cultures, enhanced the anti-SRBC response over that of lymphokine-containing supernatant alone (Table XII) (330,335). BCAF was unable to induce antibody production in the absence of lymphokine-containing supernatants. To further explore BCAF action in enhancing the anti-SRBC response, purified splenic B cells were precultured for 24 hours with BCAF-containing supernatants, washed, and then cultured with T cells and antigen. Preculturing of B cells with supernatants prepared from unstimulated spleen cells had little or no effect compared to B cells precultured in the absence of any supernatants. In contrast, B cells precultured with BCAF-containing supernatant generated a 2.5 times greater anti-SRBC response when cultured with T cells + antigen than B cells precultured in the absence of BCAF (343). None of the three B cell preparations produced an anti-SRBC response in the absence of T cells. From these data, it can be concluded that Fc fragment-induced BCAF does not directly trigger B cell differentiation, but serves as one of several B cell signals which synergize to induce antibody production and may be the major factor in the adjuvanticity of Fc fragments.

The presence of T cell-derived helper factors (TRF) involved in the differentiation process of B cells into antibody-secreting cells was

TABLE XII
ABILITY OF BCAF TO SUBSTITUTE FOR Fc FRAGMENTS IN ENHANCING THE *in Vitro* ANTI-SRBC RESPONSE BY MOUSE B CELLS[a]

Lymphokine supernatant[b]	BCAF[c]	SRBC[d]	Direct anti-SRBC PFC/10^6 cells (± SD)
−	−	+	12 ± 3
+	−	+	81 ± 1
−	+	+	19 ± 8
+	+	+	427 ± 163

[a] T cell-depleted spleen cells were cultured with SRBC ± lymphokine supernatant and BCAF and the response measured on day 4 of culture.

[b] 25 μl G-100-purified Con A supernatant.

[c] 25 μl G-100-purified Fc fragment-induced BCAF.

[d] 5×10^4 SRBC/culture.

originally described by Schimpl and Wecker (337) and Dutton (344). It was observed that TRF could be added to T cell-depleted cultures as late as 48 hours after culture initiation without decreasing the peak anti-SRBC response. These results suggested that TRF acted in the final stages of B cell differentiation. Fc fragment-induced BCAF appears to be different from TRF in that BCAF will not replace T cells but synergizes with T cell products in a fashion similar to a factor described by Swain and colleagues (345,346), derived from the long-term alloreactive line C.C3.11.75 (DL-TRF) which replaces the need for T cells in the B cell response to SRBC. Subsequently, more rigorous T cell depletion yielded B cell populations which could not be restored to anti-SRBC PFC production by DL-TRF alone, but required a second factor indistinguishable from IL-2 (347,348). In addition to synergizing with IL-2 in the anti-SRBC response model, (DL)TRF also stimulates BCL_1 cells to secrete Ig in a non-antigen-driven response model (348).

F. Enhancement of Specific *in Vitro* T Cell-Mediated Immune Responses by the Fc Fragments of Ig

Consistent with the adjuvant properties of Fc fragments being at the level of the helper T cell is the finding that antigen and alloantigen-induced T cell-proliferative responses are enhanced by Fc fragments (349). Fc fragments have the capacity to enhance ovalbumin (OVA)-

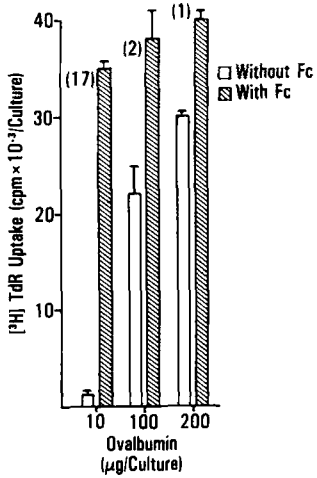

FIG. 14. Mouse lymph node-derived T cells were cultured with increasing amounts of OVA with or without 100 μg Fc/culture. Numbers in parentheses represent the enhancement index. Reprinted from Morgan *et al.* (1981). *J. Exp. Med.* **153,** 1161.

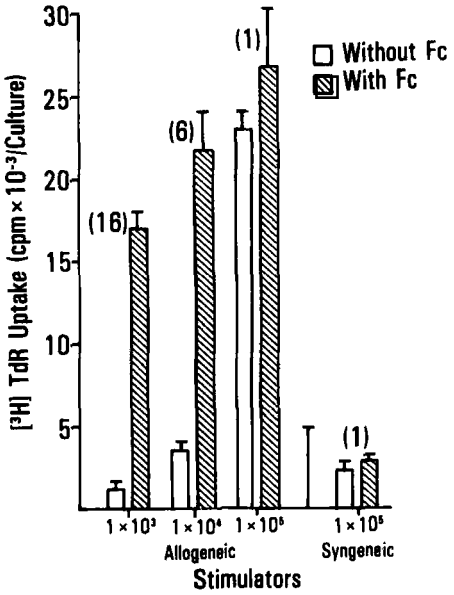

FIG. 15. C57BL/6 spleen-derived T cells were cultured with increasing numbers of irradiated CBA/CaJ spleen cells in the presence or absence of 100 μg Fc/culture. Reprinted from Morgan *et al.* (1981). *J. Exp. Med.* **153**, 1161.

induced proliferative responses with maximal enhancement occurring when suboptimal concentrations of OVA are employed (Fig. 14). It was previously reported that lymph node-derived T cells, which proliferate to antigen, belong to the Lyt-1^+2^- subclass (350). Cells bearing the Lyt-1^+2^- phenotype are also the cells affected by Fc fragments, since removal of Lyt-1^-2^+ cells has no effect on the adjuvant potential of Fc fragments (349).

In addition to enhancing antigen-induced proliferation, Fc fragments also potentiate the primary *in vitro* mixed lymphocyte culture (MLC) response between major histocompatibility locus (H-2) disparate strains of mice (349). Optimal enhancement of the primary MLC occurs with suboptimal effector : stimulator ratio (Fig. 15). Of special importance is that Fc fragment-mediated enhancement of MLC is restricted to those responses where the allogeneic differences between effector and stimulator cells encompass the I-A and/or I-B subregions. By employing B10 congeneic mice, it was observed that differences in I-A and/or I-B were mandatory for obtaining enhanced MLC (Fig. 16). The nature of the I-A and/or I-B subregion antigenic determinants does not appear to be important because Fc fragments

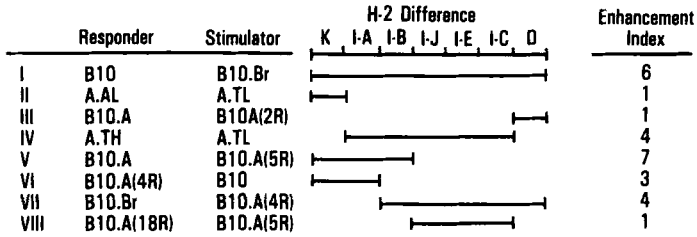

FIG. 16. Allogeneic MLC responses between mouse strains with limited H-2 differences. Suboptimal numbers (1×10^4) of stimulator cells were used. When optimal effector : stimulator ratios were used, all combinations produced an MLC ≥20,000 cpm. Reprinted from Morgan *et al.* (1981). *J. Exp. Med.* **153**, 1161.

are capable of enhancing MLC where I-A^k, I-B^k or I-A^b, I-B^b are recognized as the stimulating determinants. The Lyt phenotype of the T cells responding to I-A- and/or I-B subregion differences bears the Lyt-1^+2^- phenotype. The Lyt phenotype of the H-2D region MLC effector cells was Lyt-1^+2^+ as evidenced from the experiments where both anti-Lyt-1.2 and anti-Lyt-2.2 treatment reduced the anti-H-2D MLC to background levels.

The studies of Cantor and Boyse (351,352) indicate that the differentiation of cytotoxic T cells *in vitro* is associated with the collaboration of an amplifier or helper population with the cytotoxic T cell precursor. The amplifier T cell population appears to be expressed as the proliferating cell in the primary MLC reaction. The recognition of I-region determinants by T cells has been associated with the production of helper cells for the primary cell-mediated lympholysis (CML) response by T cells to H-2K- or D-region determinants (351–355). In addition, the helper T cell in the CML response between H-2-disparate strains bears the Lyt-1^+2^- phenotype (351,352,356,357). Taken together these results are interpreted to mean that Lyt-1^-2^+ cytotoxic T cell precursors (351,352) recognize H-2K and/or D antigens and, in conjunction with Lyt-1^+2^- cells, which recognize I region determinants, produce the CML response. Exceptions to this general model exist when limited H-2 differences (358–360), syngeneic tumors (361,362), virus-modified (363), or haptenated (364) syngeneic cells are employed. In all these latter cases it appears that an Lyt-1^+2^+ amplifier or helper cell is required for efficient expression of cytotoxicity. The work described previously (349) employing limited H-2 differences in MLC reactions confirms that of others (358–360), in that the proliferating cell population generated against H-2D-region differences is composed of Lyt-1^+2^+ cells. The reason for the ability of Fc fragments to potentiate MLC against I-A- and/or I-B-region, but not

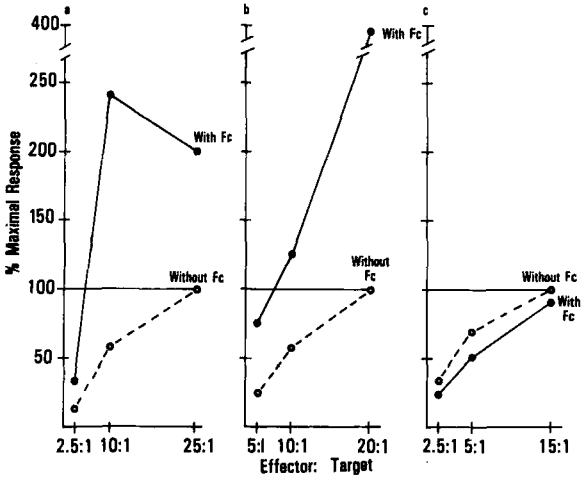

FIG. 17. Comparison of the ability of Fc fragments to augment CML responses across (a) H-2 + minor, (b) I region, and (c) D region differences; 100% maximal response is defined as the maximal killing obtained in the absence of Fc fragments. Reprinted from Morgan *et al.* (1981). *J. Immunol.* **127**, 2526.

H-2K- or D-region differences is presently unknown. Because it has been proposed that Lyt-1^+2^+ cells are precursors of Lyt-1^+2^- and Lyt-1^-2^+ cells, the possibility exists that these Lyt-1^+2^+ cells are an immature population and are unable to respond to the Fc signal. This possibility seems unlikely in light of the work by Swain *et al.* (365,366), who described the existence of long-lived, mature Lyt-1^+2^+ helper cells. These authors (365,366) found that helper activity for the primary *in vitro* response to sheep erythrocytes could be induced by recognition of foreign H-2 antigens. Helper activity generated with whole haplotype or I-region differences was supplied by Lyt-1^+2^- cells, whereas H-2K- or D-region differences activated Lyt-1^+2^+ helper cells. Since the generation of cytotoxic T cells is an extension of the MLC reaction, Fc fragments were assessed for their ability to augment CML responses (367). As described for the MLC response, Fc fragment-mediated enhancement of CML is restricted to those responses where the allogeneic difference between effector and stimulator populations encompasses the H-2I region (Fig. 17). Enhancement of the CML response occurs when differences at H-2K + I + D or H-2I regions are employed. In contrast, when strain combinations were used where only H-2D region differences occur, no enhancement is observed.

In addition to mouse *in vitro* T cell-mediated responses, Fc fragments from human Ig have been shown to enhance human T cell

proliferative responses. Passwell *et al.* (226) reported that, in the presence of indomethacin (IM), Fc fragments induced an increase in the ability of human PBM to respond to tetanus toxoid. Monomeric IgG_1, Fab fragments, and/or albumin had no effect on the immune response. Aggregated IgG human myeloma protein was also capable of enhancing tetanus toxoid-induced T cell proliferation.

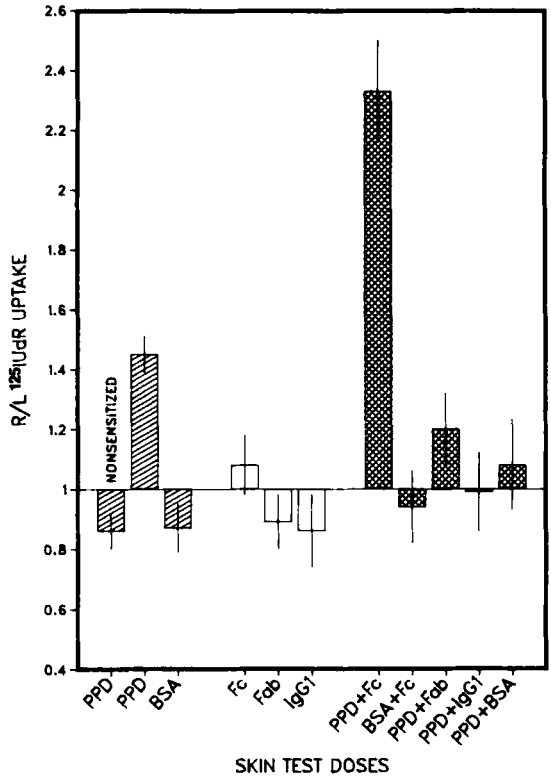

FIG. 18. Effects of Fc fragments, Fab fragments, intact IgG_1, or BSA on the DTH response to PPD. Nonsensitized rats were skin tested with 0.125 μg of PPD (NONSENSITIZED). Tubercle bacilli-sensitized rats were challenged with either 0.125 μg of PPD alone (PPD) or 0.125 μg of BSA alone (BSA). Other groups of sensitized rats were challenged with either 50 μg of Fc fragments alone (Fc), 50 μg of Fab fragments alone (Fab), or 100 μg of IgG_1 alone (IgG_1). Other sensitized groups of rats were challenged with either 0.125 μg of PPD plus 50 μg of Fc fragments (PPD + Fc), μg of BSA plus 50 μg of Fc fragments (BSA + Fc), 0.125 μg of PPD plus 50 μg of Fab fragments (PPD + Fab), 0.125 μg of PPD plus 100 μg of IgG_1 (PPD + IgG_1), or 0.125 μg of PPD plus 66 μg of BSA (PPD + BSA). Values given represent averages obtained in groups of five rats. Vertical error bars indicate limits of standard error. Inclusion of 50 μg of Fc fragments with PPD gave significantly augmented DTH responses ($p < 0.001$) when compared to the responses elicited by challenge with PPD alone. Reprinted from Ernst *et al.* (1984). *Cell. Immunol.* **89**, 445.

Fc fragments also augment T cell-mediated immunity in the rat. Ernst *et al.* (368) observed that Fc fragments from human IgG_1 potentiate rat DTH reaction to antigen challenge. Inclusion of Fc fragments with purified protein derivative of tuberculin (PPD) augmented the DTH reaction in rats immunized with complete Freund's adjuvant (Fig. 18). In contrast, equimolar concentrations of Fab fragments, intact IgG_1, or BSA did not result in an enhanced DTH reaction. Histological examination of potentiated DTH reaction sites revealed predominantly mononuclear cell infiltrates characteristic of DTH reactions. The mechanism(s) of Fc fragment-mediated potentiation of DTH responses is unknown. Since Fc fragments enhance the DTH response to the relevant antigen but do not elicit appreciable responses by themselves in immunized animals, they may be acting on the cells activated and/or recruited by the challenge antigen. Fc fragments could work in conjunction with antigen in modulating the activity of DTH-T cells which bear the helper T cell phenotype (369). In the rat, an analogous helper/inducer T cell subpopulation is defined by the W3/25 monoclonal antibody (370), which includes T cells that mediate DTH to PPD (371). It is possible that murine and rat helper T cells are similarly affected by Fc fragments of Ig.

G. Stimulation of Macrophages by Fc Fragments of Ig

IC, AHGG, and Fc fragments of Ig have been reported to activate animal macrophages and human monocytes to secrete a number of biologically active mediators (226,228,301,372–380). Soluble mediators derived from monocytes/macrophages are known to participate in suppression and/or enhancement of antigen- and mitogen-induced immune responses (381). Considerable evidence exists showing that the monocyte/macrophage is a major source of oxidative metabolic products of AA such as PG (382,383). PG have been shown to be potent inhibitors of both humoral and cell-mediated immunity (229,230).

AA, liberated from membrane-bound phospholipids by PLA_2, is oxidized via two general pathways (Fig. 19). The cyclooxygenase pathway converts AA into PG, prostacyclins (PGI), and thromboxanes (Tx), whereas the lipoxygenase pathway results in the production of leukotrienes (LT) and the hydroperoxy- and hydroxyeicosatetranenoic acid intermediates (HPETE and HETE) (384). In addition to their phlogogenic properties, attention has recently been focused on the suppressive properties of AA metabolites (229,230). Smith *et al.* (385) first reported that PGE_1, PGE_2, PGA, and $PGF_{1\alpha}$ inhibit mitogen-induced T cell proliferation. Subsequently, other investigators have

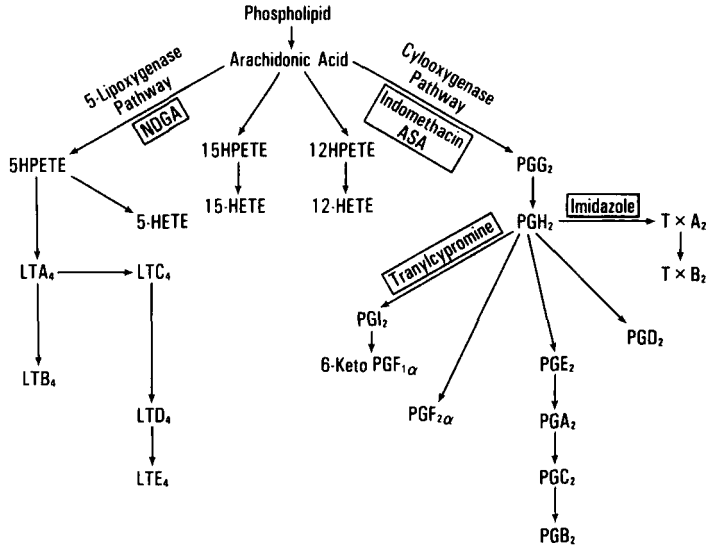

FIG. 19. Pathways of arachidonic acid metabolism.

reported that PGE_1 and/or PGE_2 depresses (1) antibody formation (377,386), (2) hemolytic plaque formation (387), (3) cytolytic activity of activated lymphocytes (388), (4) macrophage inhibitory activity (389), (5) IL-2 production (390,391), (6) Ia expression (392), (7) IL-1 production (393), and (8) mitogen-induced B cell transformation (394). Passwell and co-workers (226,373) originally reported that the coculture of Fc fragments of IgG with human monocyte monolayers resulted in a marked increase in PGE release by the monocytes. Fab fragments, monomeric IgG_1, and human serum albumin did not induce PGE release. Furthermore, and IgG_1 myeloma protein had no effect on PGE release unless it was heat aggregated prior to culturing with monocytes. The increase in PGE secretion resulting from exposure of the monocyte monolayers to Fc fragments greatly exceeded the increases observed after the addition of antigen-activated mononuclear cell supernatants, zymosan, Sephadex beads, or LPS. These authors (226) observed that PGE_2 accounted for approximately 70% of the total PG released by stimulated cells. Inclusion of indomethacin (IM) in Fc fragment-containing cultures resulted in an enhanced proliferative response to tetanus toxoid. Omission of IM resulted in a suppressed proliferative response to tetanus toxoid. Fc fragments were also found to induce polyclonal production of IgM by PBM and tonsillar B cells in the presence of IM (226). Thus, in general the Fc

TABLE XIII[a]
Fc Fragment-Induced PGE Release from Mouse Splenic Macrophages

Fc fragments (μg/ml)	PGE (ng/ml)[b]
—	0.7
1	1.8
10	3.0
100	5.4
1000	5.5

[a] Reprinted from Morgan *et al.* (1985). *J. Immunol.* **134**, 2247.
[b] Supernatants were assayed for PGE by RIA after 24 hours of culture.

fragment acts as an adjuvant, but through the activation of AA cascade with subsequent release of PG, can down regulate either this enhancement or the immune response itself.

The bifunctional regulatory nature of the Fc fragment was further substantiated by our laboratory (301,377). Fc fragments were found to induce the release of PGE from murine splenic macrophages (Table XIII). Maximum PGE release was achieved with approximately 100 μg Fc/ml. PGE release was specific for Fc fragments because Fab fragments and monomeric IgG_1 were unable to induce PGE release (377). Fc fragments also induce the $P388D_1$-macrophage-like cell line to release PGE (Table XIV). These results confirm that macrophages are one source of PGE in this model. To determine whether endogenous PGE release could be adversely affecting mouse *in vitro* polyclonal Ig production, spleen cells were cultured with Fc fragments in

TABLE XIV
Fc Fragment-Induced PGE Release from $P388D_1$ Cells[a]

	PGE (ng/ml)	
Stimulator	Experiment 1	Experiment 2
—	<0.027	<0.027
Fc	0.31	2.5
Fab	<0.027	ND
IgG_1	<0.027	ND

[a] Supernatants were assayed for PGE by RIA after 24 hours of culture.

the presence or absence of IM, a PG synthesis inhibitor (395), and the supernatant Ig concentrations were measured. The results shown in Fig. 20 indicate that IM is capable of enhancing Fc fragment-mediated polyclonal Ig secretion, presumably through inhibition of PG synthesis.

IM has also been shown to augment a variety of mitogen-induced proliferative responses (229,230). Because Fc fragments induce mouse B cells to proliferate and induce macrophages to release PGE, studies were conducted to determine if IM could enhance Fc fragment-induced B cell proliferation. In contrast to its effects on polyclonal Ig secretion, addition of IM to culture did not enhance B cell proliferation (377). These results suggest that, in Fc fragment-induced Ig secretion, PG acts late in blocking the response. PG could prevent Ig secretion by blocking production and/or action of Fc fragment-induced BCAF.

Fc fragments of human IgG_1 also induce human PBM-derived monocytes to release PGE (Fig. 21), confirming earlier observations (226). Fc fragments, under the experimental conditions employed, preferentially activate the cyclooxygenase pathway of AA metabolism.

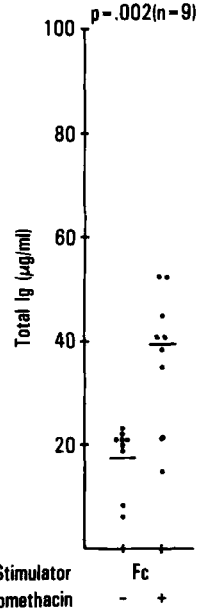

FIG. 20. Murine spleen cells were cultured with 166 μg Fc fragments/ml with or without 5 μg IM/ml. Horizontal bars represent the mean value. Reprinted from Morgan *et al.* (1985). *J. Immunol.* **134**, 2247.

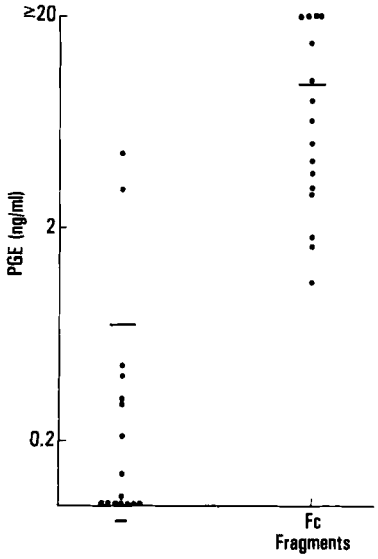

FIG. 21. Peripheral blood-derived monocytes were cultured with 10 μg Fc fragments/ml and the culture supernatants assayed for the presence of PGE by RIA after 24 hours. Each point represents an individual experiment.

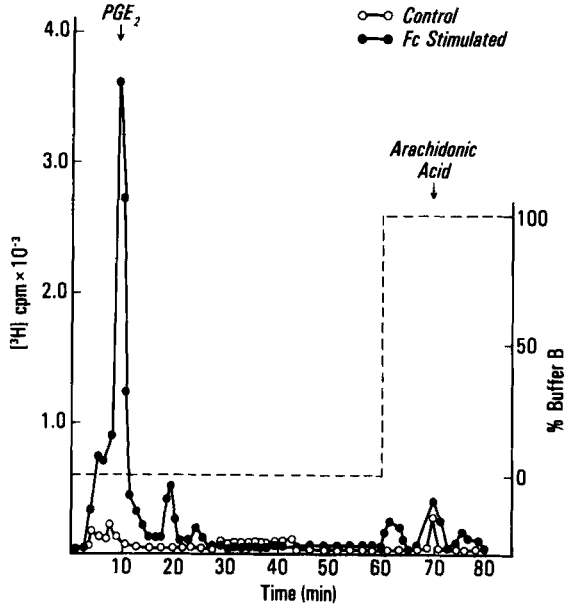

FIG. 22. C_{18} HPLC separation of culture supernatants from [^{3}H]AA-labeled monocytes after a 24-hour stimulation with 10 μg Fc fragments/ml.

Reverse-phase (C_{18}) high-performance liquid chromatographic (HPLC) separation of Fc–monocyte supernatants revealed the presence of a number of AA metabolites (Fig. 22). Separation of monocyte supernatant revealed the presence of one major peak (R_T = 8 minutes) and four smaller peaks with R_T of 18, 24, 62, and 70 minutes. C_{18} HPLC elution of PGE_2 standard resulted in a peak with an R_T of approximately 8 minutes. Since C_{18} does not adequately resolve cyclooxygenase pathway metabolites (228), 0- to 10-minute elution material was collected, concentrated, and rechromatographed on a fatty acid analysis column (FAAC). This procedure resolved the 0- to 10-minute fractions into four major peaks and a number of minor peaks. Comparison of R_T with standards revealed that Fc fragment-stimulated PBM supernatants contained material coeluting with TxB_2 (R_T = 23 minutes). $PGF_{2\alpha}$ (R_T = 29 minutes), and PGE_2 (R_T = 37 minutes) (Fig. 23). Peaks coeluting with R_T of 3, 14, and 48 minutes were also observed but are presently unidentified. These results extend the data obtained by RIA showing the presence of PGE_2 in supernatants from Fc fragment-stimulated PBM cultures to include other oxidative metabolic products of AA.

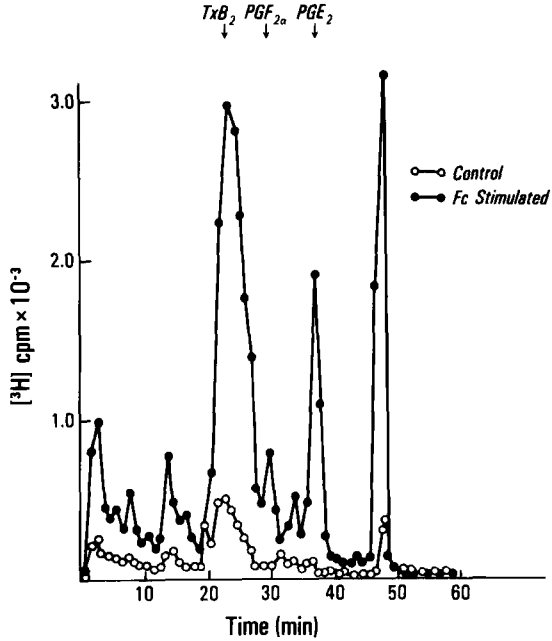

FIG. 23. Fatty acid analysis column HPLC separation of fractions 0–10 from Fig. 22.

In addition to activating the cyclooxygenase pathway, IC have recently been shown to activate the lipoxygenase pathway of AA metabolism (228). Recent experiments from this laboratory indicate that Fc fragments from human IgG_1 induce human monocytes to release LTB_4 (Table XV), and the 5-lipoxygenase pathway intermediate 5-HETE.

The mechanisms by which endogenous PG regulate Fc fragment-induced Ig secretion are unknown. PG may directly affect lymphocyte function or affect immune responses indirectly by altering accessory cell function. PGE_2 has been reported to inhibit mitogen-induced B and T lymphocyte proliferation (229,230). Thompson *et al.* (394) observed that PGE_2 suppresses B cell proliferation. However, experiments performed in the murine model indicated that the addition of PGE_2 or IM to culture failed to alter Fc fragment-induced B cell proliferation (377). A second possibility is that PG is interfering with T cell activation and/or the generation of a T cell-derived B cell differentiation signal. Thoman *et al.* (305–307) have shown that polyclonal Ig secretion induced by Fc fragments is dependent on T cells and that these fragments induce T cells to secrete BCAF. This potential mode of PG-mediated inhibition is consistent with reports showing that PG is capable of directly or indirectly suppressing the synthesis and/or action of IL-2 (390,391,396–398). PG has also been reported to inhibit the production of interleukin-1 (IL-1) (393), a possible regulator of IL-2 synthesis. A third possibility is the ability of PGE_2 to disrupt lymphocyte–accessory cell interaction through the down regulation of class II major histocompatibility antigen expression on accessory cells (392).

TABLE XV
Fc Fragment-Mediated Activation of the Lipoxygenase Pathway of Arachidonate Metabolism

Human PBM[a]	Fc fragments (μg/ml)	LTB_4[b] (pg/ml)	
		Experiment 1	**Experiment 2**
+	—	310	110
+	0.1	370	ND[c]
+	1.0	650	ND
+	10.0	900	380
+	50.0	ND	450

[a] 5×10^6 PMB.

[b] Supernatants were collected after 1 hour incubation and assayed for LTB_4 by RIA.

[c] ND, Not done.

It was previously reported (292) that intact PBM cultures were inconsistent in their ability to produce Ig in response to stimulation with Fc fragments. This problem was alleviated by irradiating the T cells prior to their addition to B cell-enriched populations. Recently, we have shown (301) that intact PBM cultures will consistently secrete Ig when stimulated by Fc fragments in the presence of PG synthetase inhibitors. Thus, the inconsistency in inducing polyclonal Ig secretion in intact cultures with Fc fragments could be due to the concomitant release of suppressive PG.

Additional support for a role of Fc region fragments in the activation of the AA cascade comes from the observations of Suzuki and co-workers, who reported that FcR on macrophages possess PLA_2 activity (232–234,399), an enzyme needed for the liberation of AA from membrane-bound phospholipids. The activation of PLA_2 is therefore regarded as the initial rate-limiting step of the biosynthesis of PG and LT. Suzuki and co-workers observed that FcR for IgG isolated from human leukemic B cells and the FcR for IgG_{2b} isolated from $P388D_1$ cells contained an enzyme with properties similar to PLA_2 (i.e., activity at alkaline pH, Ca^{2+} dependency, and specificity for the ester bond at the C_2 position of the glycerol backbone of phospholipids). These authors (232) speculate that antigen–antibody complex-mediated down regulation of immune function could be through binding of FcR with subsequent activation of PLA_2 and release of suppressive PG.

In addition to metabolites of AA, other macrophage products also play a role in immunoregulation. IC, AHGG, and Fc fragments stimulate monocytes/macrophages to secrete biological factors, such as IL-1, which potentiate immune function (378,379). IL-1 has been shown to influence a variety of nonimmunologic events, such as induction of fever (400), stimulation of acute-phase protein synthesis (401), induction of neutrophilia (401), modulation of serum cation concentration (401,402), and activation of AA metabolic pathways (230). In addition, IL-1 contributes to host defense in that it enhances various *in vitro* immune responses, including T cell proliferation through induction of IL-2 (403), B cell differentiation (404), and antibody secretion (405). More recently, IL-1 has been shown to enhance *in vivo* secondary antibody responses in mice to soluble protein antigens (406). Thus, IL-1 may be responsible in part for the stimulatory effects of most adjuvants.

Blyden and Handschumacher (378) first reported that soluble antigen–antibody complexes could induce cultured human monocytes to release IL-1. IC were found to be as effective as other known macrophage activators in inducing IL-1 secretions. Similarly, Dayer *et al.*

(379) reported that adherent synovial cells derived from synovia of patients with rheumatoid arthritis produced high levels of IL-1-like activity (mononuclear cell factor, MCF) upon stimulation with Fc fragments of human IgG or AHGG, whereas Fab fragments were inactive. Although AHGG and Fc fragments stimulate PGE_2 synthesis and secretion by human monocytes, IL-1 production is not induced by PGE_2 since concentrations of IM that completely block PGE_2 production do not inhibit IL-1 production. These authors (379) speculate that increased production of IL-1 by monocytes in response to substances that probably exert their effects via surface receptors could be relevant in interpreting the *in vivo* role of IC in disease states. The ability of Fc fragments of Ig and IC to stimulate monocytes to secrete IL-1 has recently been substantiated by us. Incubation of human adherent monocytes with Fc fragments but not Fab fragments resulted in the appearance of IL-1-like activity in culture supernatants (Table XVI). To determine the approximate M_r of the IL-1-like activity, adherent cell supernatants were fractionated by molecular exclusion HPLC. The results in Fig. 24 reveal that thymocyte comitogenic activity is associated with an M_r of approximately 15,000. Absence of IL-2 in the culture supernatants was shown by their inability to stimulate the IL-2-dependent cell line, CTLL-2 (E. Morgan, unpublished observations). More recently, Fc fragments have been shown to stimulate $P388D_1$ cells to secrete a factor capable of stimulating mouse thymocytes to proliferate in the costimulator assay. In contrast to these results, others have been unable to induce IL-1 secretion by IC and

TABLE XVI
Fc Fragment-Mediated Release of IL-1 from Human Monocytes[a]

Thymocytes	PHA	Monocyte supernatant[b]	[^{3}H]TdR uptake (cpm ± SD)
+	−	—	687 ± 21
+	+	—	469 ± 1
+	+	Control	2,253 ± 462
+	+	Fc (0.1 μg/ml)	12,787 ± 7,078
+	+	Fc (1.0 μg/ml)	45,120 ± 9,432
+	+	Fc (10.0 μg/ml)	73,320 ± 10,583
+	+	Fab (10.0 μg/ml)	1,719 ± 156

[a] Supernatants derived from monocyte cultures were assayed in the mouse thymocyte costimulator assay.

[b] Monocytes were cultured with Fc or Fab fragments for 24 hours prior to collection of culture supernatants.

Fc fragments in the absence of C (407). However, C fixation by precipitated IC did induce monocytes to secrete IL-1. They also reported (407) that supernatants from monocytes cultured on adherent IC contained a factor which inhibited IL-1 generation. These authors speculated that previous reports (378,379) of IC- and Fc fragment-induction of IL-1 were due to low level endotoxin contamination and that neither Fc fragments nor IC, in the absence of C or endotoxin, were stimulatory. In contrast, Dayer *et al.* (379) determined that LPS was not responsible for Fc fragment- and AHGG-induced IL-1 secretion in their system by showing that inclusion of polymyxin B sulfate into culture did not inhibit stimulatory effects of these reagents. The complexing of polymyxin B with the lipid A portion of LPS abolishes many of the biological activities associated with LPS (408–410). Recently, Doe *et al.* (408) observed that a protein-rich component noncovalently bound to lipid A (lipid A-associated protein, LPA) was capable of activating macrophages into killer cells. Moreover, biological activity associated with LPA was not inhibited by polymyxin B. These results indicate that not all biological activities associated with

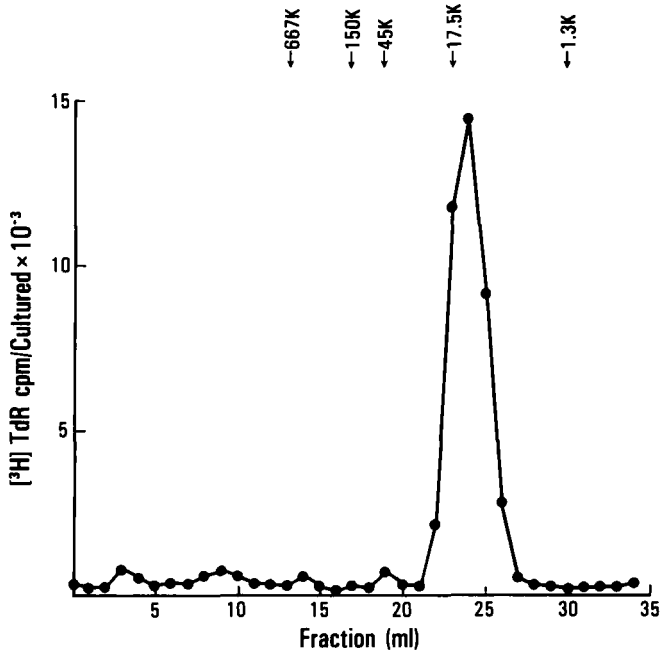

FIG. 24. Molecular exclusion HPLC separation of culture supernatants derived from incubation of human monocytes and 10 μg Fc fragment/ml. Each HPLC fraction was assessed for IL-1 activity in the thymocyte costimulator assay.

LPS are inhibited by polymyxin B. Whether LPA induces IL-1 secretion from macrophages is unknown; however, lipid A has been shown to be a potent inducer of IL-1 secretion (revue: ref 393). Results from our laboratory indicate that although Fc and Fab fragments from IgG_1 contain trace amounts of LPS (≤ 10 ng/mg protein), only Fc fragments are capable of inducing monocytes to secrete IL-1. The mechanism by which IC and Fc fragments of Ig induce monocytes/macrophages to secrete IL-1 is currently unknown. One possible mechanism is through activation of the lipoxygenase pathway of AA metabolism. Dinarello *et al.* (411) have recently provided indirect evidence for the involvement of the lipoxygenase pathway in IL-1 production. These authors (411) observed that lipoxygenase pathway inhibitors could block the production of IL-1. More recently, Rola-Pleszczynski and Lemaire (412) reported that LTB_4 and LTD_4 enhanced IL-1 production by LPS. Adherent human monocytes could also be induced to secrete IL-1 in response to LTB_4 and LTD_4. These findings are of special importance in light of the observation that IC (228) and Fc fragments of IgG (E. Morgan, unpublished observations) activate the lipoxygenase pathway of AA metabolism. Rouzer *et al.* (228) showed that mouse peritoneal macrophages released LTC_4 after exposure to particulate IgE and IgG containing IC. Exposure of human monocytes to Fc fragments resulted in the appearance of LTB_4 in culture supernatants at 1–5 hours (Table XV). It was also observed that the addition of synthetic LTB_4 (gift of Dr. J. Rokach, Merck Frosst) to monocyte cultures resulted in the production of IL-1 (Table XVII),

TABLE XVII
LEUKOTRIENE-INDUCED IL-1 RELEASE FROM HUMAN MONOCYTES[a]

Thymocytes	PHA	Monocyte supernatants[b]	[^{3}H]TdR uptake (cpm ± SD)
+	−	—	392 ± 144
+	+	—	407 ± 19
+	+	Control	9,577 ± 503
+	+	LTB_4 (1.0 ng/ml)	31,543 ± 105
+	+	LTB_4 (10.0 ng/ml)	20,718 ± 747
+	+	LTB_4 (100.0 ng/ml)	15,536 ± 5,699

[a] Supernatants derived from monocyte supernatants were assayed in the mouse thymocyte costimulator assay.

[b] Monocytes were cultured with LTB_4 for 24 hours prior to collection of the supernatants.

thus confirming the findings of Rola-Pleszczynski and Lemaire (412).

Activation of the lipoxygenase pathway of AA metabolism by IC and Fc fragments of Ig with subsequent production of IL-1 could partially account for the ability of these agents to enhance immune responses. Recent studies suggest that inhibition of the lipoxygenase pathway with nordihydroguaniacetic acid (NDGA) inhibited *in vitro* CMI responses (413). In addition, others have reported that optimal NK cell activity may require lipoxygenation of AA. It was observed that both human and mouse NK cell activity were reversibly inhibited by NDGA and BW755C, another lipoxygenase pathway inhibitor (414). These findings indirectly suggest a role for IL-1 in NK cell activity.

H. Biologically Active Peptides from the Fc Region of Ig

Proteolytic cleavage of Ig has long been used as a basic method for the localization and characterization of biologically active regions within the Ig molecule. Enzymes such as papain, pepsin, trypsin, elastase, and plasmin have been used to cleave the Ig molecule into various size fragments exhibiting different biological activities (see Section IA). In addition to the classical effector functions, there is growing evidence indicating that peptides derived from Ig (300,415–422) and non-Ig (170,423–425) sources possess immunoregulatory activity.

Najjar and co-workers (426–428) originally reported the existence of a small M_r peptide derived from enzymatic cleavage of IgG. The peptide, tuftsin, was derived from the CH_2 domain of IgG (residues 289–292; Thr-Lys-Pro-Arg). Cleavage of Ig to produce tuftsin has been shown to require the participation of two enzymes. One cleaves between residues 292 and 293 and is termed tuftsin carboxy peptidase (428). The other enzyme, leukokinase, cleaves between residues 288 and 289 (415,428). Tuftsin has been reported to induce and/or influence a wide variety of biological responses (Table XVIII). Tuftsin appears to act by binding to receptors on the target cell populations. Fridkin and co-workers (440,442,443), using radiolabeled tuftsin, reported the existence of predominantly one type of receptor on mouse macrophages and human granulocyte for tuftsin. Tuftsin receptors have also been reported on lymphocytes (444) and NK cells (415).

Structure–function and conformational studies of the tuftsin molecule predicted that another peptide, Gly-Gln-Pro-Arg (Eu IgG_1, sequence 341–344), would contain "tuftsin-like" activity (419). This peptide, termed rigin, was synthesized and found to exert phagocytosis-stimulating activity toward heterologous erythrocytes and bacteria.

TABLE XVIII
BIOLOGICAL ACTIVITIES ASSOCIATED WITH TUFTSIN

Function	Reference
Chemotaxis	429
Phagocytosis	430,431
Increase antigen processing by macrophages	432,433
Increase antibody production	434,435
Induce colony formation	436
Tumor cytostasis	434
Enhance cytotoxic T cell killing	437
Enhance antibody-dependent cytotoxicity	438
Enhance natural killer cell activity	416
Bactericidal	439
Modulate cyclonucleotide levels	440
Activate arachidonate cascade	441

On a molar basis, rigin was found to have a potency similar to tuftsin in enhancing phagocytosis by macrophages.

In addition to tuftsin and rigin, other investigators have reported the existence of biologically active peptides derived from enzymatic cleavage of Ig (418,445–451). Higuchi *et al.* (445) reported that a dialyzable peptide released from rabbit IgG after 3 to 6 hours digestion with rabbit neutrophil neutral thiol protease exhibited a distinct chemotactic activity for B lymphocyte from the thoracic duct lymph and spleen of rats. Moreover, these authors (445) observed that the chemotactic peptide could be generated from Fc fragments but not Fab fragments of rabbit IgG. Neutral serine protease, in addition to thiol protease, has recently been shown to generate macrophage chemotactic factors from IgG (452). Capron and colleagues (418,453) have demonstrated two enzymes derived from *Schistosoma mansoni* larvae, neutral endoprotease of the serine group and neutral metalloaminopeptidase, cleave surface-bound IgG into peptides capable of suppressing antibody-dependent macrophage cytotoxicity against *Schistosomula.* More recently, these authors (453) observed that the tripeptide, Thr-Lys-Pro, derived from the CH_2 domain of human IgG_1 (residues 289–291), mimicks the inhibitory effects of the Ig digested by parasite enzymes. In addition, Thr-Lys-Pro was found to inhibit β-glucuronidase release, chemiluminescence, and IL-1 production by rat macrophages. These authors speculate that IgG peptide-induced down regulation of macrophage function could represent part of the parasite resistance mechanism. The sequence Thr-Lys-Pro has been shown by other investigators to be an antagonist of tuftsin (426).

Murine and human B cells are induced to secrete polyclonal Ig when cultured with AHGG and IC and Fc fragments derived from papain, plasmin, or pepsin digestion of Ig (see Section II,D). A unifying feature of these responses is that in each case a portion of the constant region of the Ig heavy chain (i.e., Fc portion) is present. $F(ab')_2$ and Fab fragments proved to possess no stimulatory activity. Another feature of polyclonal activation by these agents is that macrophages/monocytes are required as accessory cells. Macrophage/monocytes appear to enzymatically cleave the Ig molecule resulting in the release of biologically active Fc subfragment (299) (see Section II,D). To define the active sequence from the Fc region of IgG_1, a fragment containing approximately the CH_3 domain was degraded with cyanogen bromide (CNBr) and the resulting peptides assayed for their ability to stimulate mouse B cells to secrete polyclonal Ig. The IgG_1 human myeloma protein (Pet) used in these studies bears the *Gm*^{f} allotype markers with methionine (Met) at position 358 (454). An additional Met is also present at position 428 (454). Cleavage of the biologically active plasmin-digested fragment of IgG_1 at the Met residues with CNBr, with subsequent reverse-phase HPLC separation, resulted in the isolation of two small peptides (Fig. 25). Analysis of pool A and B for biological activity revealed that the ability to induce a polyclonal Ig secretion was associated with pool A.

The amino acid composition of pool A identified this material as residues 335–358 in Eu IgG_1 (455). The peptide in pool B corresponded to residues 429–446 in the Eu IgG_1 sequence. It can be concluded from these results that at least one region of the Ig molecule responsible for inducing polyclonal Ig secretion is associated

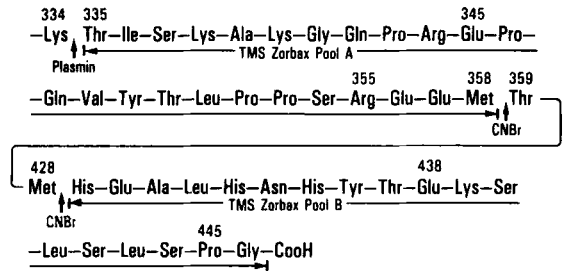

FIG. 25. A schematic representation of residues 334–445 of the Eu IgG_1 myeloma protein. Upon treatment with CNBr, three peptide pools are produced. Pool A consists of residues 335–358 and contains all the biological activity. Pool B consists of the carboxyterminal peptides, residues 429–445, and is unable to induce polyclonal antibody production. Reprinted from Morgan *et al.* (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5388.

with residues 335–358 located at the amino terminal end of the CH_3 domain. It is of interest to note that the peptide rigin described above is located within this peptide. Whether the biologically active peptides described by this laboratory enhance phagocytosis (vis a vis, tuftsin and rigin) is unknown and must await further investigation.

Solid phase synthesis of the biologically active region comprising residues 335 to 358 in the Eu IgG_1 sequence resulted in a peptide, p23, which contains a number of the biological properties previously associated with Fc fragments, AHGG, and IC (299). Induction of polyclonal Ig secretion in mouse spleen cell cultures was found to be dose dependent, and cultures reached maximal Ig levels by day 4 to 5 of culture (300,456). In this system, B cell activation by Fc fragments has been shown to be both macrophage and T cell dependent (299). Assessment of cellular requirements for polyclonal Ig production by p23 revealed that removal of T cells dramatically reduced the amount of Ig present in culture supernatants (Table XIX). In contrast, depletion of macrophages only marginally affected polyclonal Ig secretion. These data are consistent with previous reports and suggest that p23 may represent one of the active sites exposed on the Fc fragment after the necessary processing by macrophages.

Based on the findings that Fc fragments, AHGG, and IC activate AA metabolic pathways (226,301,377,373), p23 was assessed for its ability to induce PGE release from mouse macrophages. Coculture of p23 with mouse splenic macrophages resulted in the release of significant amounts of PGE into culture supernatants compared to unstimulated cultures (Table XX) (377). With the finding that the tuftsin-like pep-

TABLE XIX[a]
REQUIREMENT FOR T CELLS IN p23-INDUCED POLYCLONAL ANTIBODY SYNTHESIS

Cell treatment	Stimulator	Micrograms Ig/milliliter[b]		
		Experiment 1	Experiment 2	Experiment 3
None	—	<1	2	11
None	p23[c]	150	39	40
None	LPS[d]	187	31	50
T cell depleted	—	<1	4	3
T cell depleted	p23	32	15	7
T cell depleted	LPS	138	35	60

[a] Reprinted from Hobbs *et al.* (1985). *J. Immunol.* **134,** 2847.
[b] Supernatants were assayed for Ig levels by direct ELISA.
[c] 1.4×10^{-4} μM/ml.
[d] 30 μg/ml.

TABLE XX[a]
PGE RELEASE BY MOUSE SPLENIC MACROPHAGES[b]

Stimulator	μg/ml	PGE (pg/ml)
—	—	700
p23	0.01	2600
p23	0.1	2650
p23	1.0	5400
p23	10.0	7000

[a] Reprinted from Morgan (1985). *J. Immunol.* **134**, 2247.

[b] Mouse splenic macrophages were cultured with p23 for 24 hours prior to collection of supernatants.

tide rigin (419) is located within p23 (341–344), and that tuftsin (and perhaps rigin) is known to activate the cyclooxygenase pathway of AA metabolism (441), it is tempting to speculate that this site may be responsible for inducing PGE release from macrophages. However, synthetic peptides containing rigin, but not the active site for polyclonal Ig secretion, do not cause PGE release (see below). It is possible that for rigin to be active it must be a free peptide and when incorporated into p23 or p23 analog peptides it is rendered inactive.

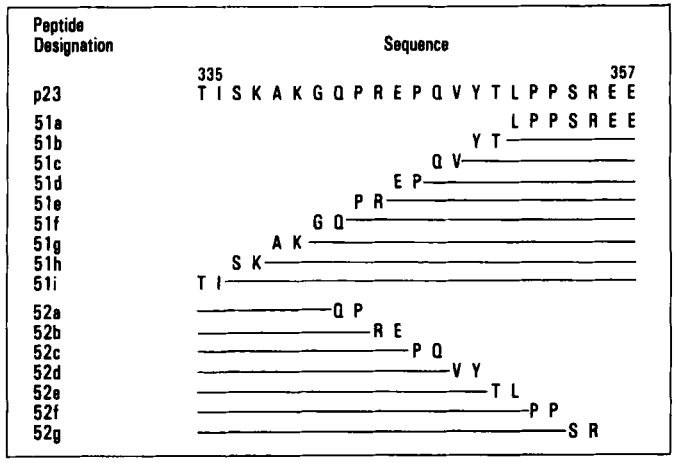

FIG. 26. Sequences of 51 and 52 series p23 subpeptides. Reprinted from Hobbs *et al.* (1986; see Table 21).

To determine the minimal sequence in p23 responsible for the induction of B cell differentiation, peptides of various lengths originating from either the carboxy or the amino terminus of p23 were synthesized (421) (Fig. 26) and assessed for biological activity. It was observed that peptides increasing in length from the amino terminus (52 series) did not exhibit any biological activity until Ser_{354} and Arg_{355} were added (Fig. 26). Analysis of peptides synthesized from the carboxy terminus (51 series) revealed that peptides 51a, 51b, and 51c also exhibited the ability to induce mouse spleen cells to secrete polyclonal Ig (Fig. 27). Peptide 51a, originating from the carboxy terminus, is the smallest peptide retaining biological activity. Analysis of the data with overlapping peptides suggests that the minimal active sequence needed for B cell differentiation is Leu_{351}-Pro_{352}-Pro_{353}-Ser_{354}-Arg_{355}. An interesting finding is that certain 51 series peptides that contain Leu-Pro-Pro-Ser-Arg (51d, 51e, 51f, 51g, 51h) were unable to stimulate mouse B cells to secrete polyclonal Ig (Fig. 27). One possible explanation for this finding is that the 51d–h peptides assume a conformation not conducive to the expression of the biologically active site. To test this hypothesis, attempts were made to generate active peptides by chymotyrpsin digestion of the biologically inactive 51g peptide. Chymotrypsin cleavage of 51g at the aromatic residue 349 results in the production of at least two smaller peptides. Chymo-

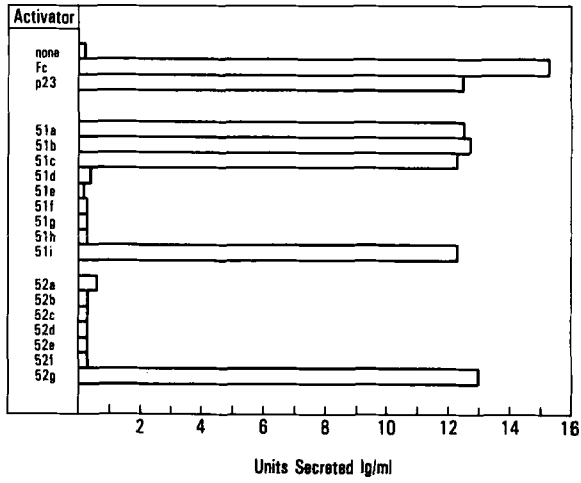

FIG. 27. Induction of polyclonal antibody production by synthetic p23 subpeptides. Mouse spleen cells were stimulated with Fc fragments (75 μg/ml), or synthetic peptides shown in Fig. 26, each at a dose equimolar to p23 (1.5 μmol/ml). IM (5 μg/ml) was present in all cultures. Reprinted from Hobbs *et al.* (1986; see Table 21).

TABLE XXI[a]
INDUCTION OF POLYCLONAL ANTIBODY PRODUCTION BY CHYMOTRYPSIN-DIGESTED 51g

Activator	Secreted Ig (μg/ml)
None	2.8
Chymotrypsin-digested 51g	14.9
Sham-digested 51g	3.0
Chymotrypsin control	2.5

[a] Reprinted from Hobbs *et al.* (1986). *In* "Immune Regulation by Defined Polypeptides" (G. Goldstein, J. F. Bach, and H. Wigzell, eds.). Liss, New York (in press).

trypsin digestion resulted in a mixture which was able to induce mouse spleen cells to secrete Ig, whereas the sham-digested peptide and chymotrypsin control were inactive (Table XXI).

Based on the observation that p23-induced polyclonal Ig secretion requires T cells (300,457), p23 was assessed for its ability to induce T cells to secrete BCAF. Coculture of mouse spleen cells and p23 for 24 hours resulted in the appearance of BCAF in the culture supernatants. Reverse-phase (Fig. 28) and gel permeation (Fig. 29) HPLC purification revealed that biological activity was contained in a single peak. HPLC-purified p23-BCAF was found to be devoid of BCGF I, IL-2, and IL-1 activity (M. Thoman, personal communication). These results indicate the p23- and Fc fragment-induced B cell activation occur through similar pathways.

Peptides (51 and 52 series) were also assessed for their ability to stimulate mouse splenic macrophages to secret PGE. The data shown in Fig. 30 indicate that only 51i (equivalent to p23) and 52g (p23 minus the carboxy terminal Glu_{356} and Glu_{357}) were able to induce the release of PGE from plastic-adherent splenic macrophages. Stimulation of the $P388D_1$-macrophage-like cell line with equimolar concentrations of 51 and 52 series peptides revealed that, like splenic macrophages, only 51i and 52g are able to induce the release of PGE. These results indicate that the induction of polyclonal Ig secretion and release of PGE by mouse cells have different sequence requirements within the p23 molecule.

p23 has been shown to activate human B cells to secrete polyclonal Ig in a manner analogous to Fc fragment-induced activation (301). These results support data obtained in the mouse model, indicating that at least one biologically active region of the Fc fragment is con-

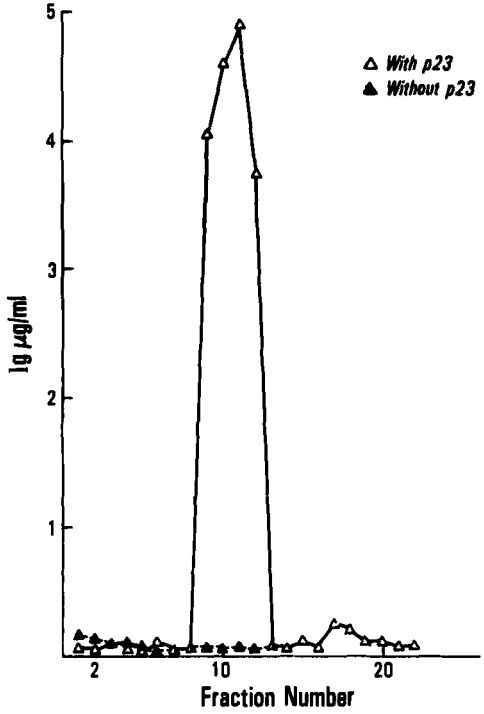

FIG. 28. C_{18} reverse-phase HPLC separation of 24 culture supernatants derived from the cocultures of mouse spleen cells and p23. Each HPLC fraction was assessed for BCAF activity in T cell-depleted mouse spleen cell cultures.

tained within p23. Furthermore, mapping of the minimal sequence needed for human B cell activation and PGE release with the 51 and 52 series peptide reveals that Leu-Pro-Pro-Ser-Arg is needed for polyclonal Ig production and p23 or p23 minus carboxy terminal residues 356 and 357 is needed to induce monocytes to release PGE.

Fc fragment-induced B cell proliferation (301) and IL-1 secretion (421) are not associated with the amino acid sequence comprising p23. Coculture of human monocytes or the $P388D_1$ cell line with p23 does not result in the release of measurable amounts of IL-1. Table XXI summarizes biological activities associated with the Fc region of Ig. B cell differentiation, BCAF secretion, and activation of AA cascade all map to the amino terminus of the CH_3 domain of human IgG_1. To date, induction of B cell proliferation and IL-1 secretion have only been observed with IC, AHGG, and Fc fragments derived from enzymatic cleavage of IgG. Work is currently in progress in defining the minimal regions needed for inducing B cell proliferation and IL-1 secretion.

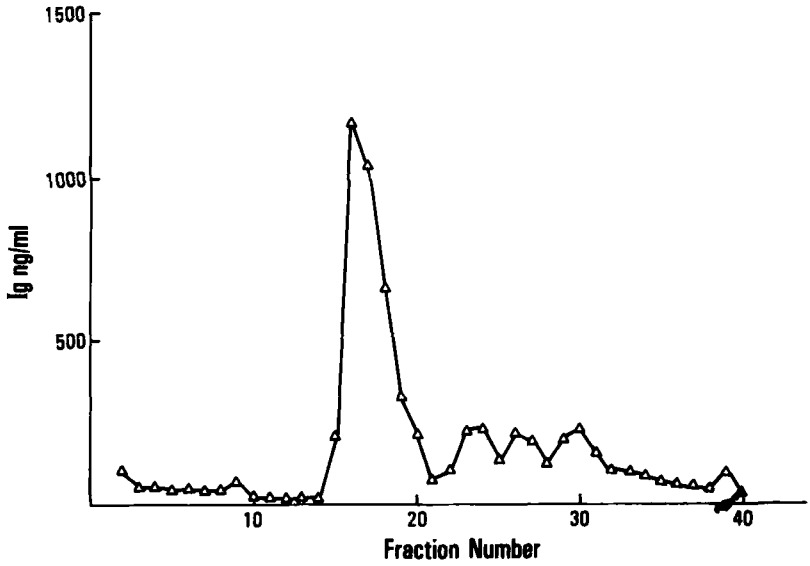

FIG. 29. Active C_{18} fractions were subjected to gel permeation HPLC separation. Each HPLC fraction was assessed for BCAF activity in T cell-depleted mouse spleen cell cultures.

The ability of Fc fragments and subfragments to release a number of biologically active mediators (Table XXII), which include BCAF, IL-1, PG, and LT, demonstrates the complexity of Fc fragment- and subfragment-mediated immunoregulation. Based on the results obtained in mapping the various biological functions to sites within the Fc molecule, the potential exists to tailor a synthetic Ig peptide to include or exclude certain biological activities.

III. Summary

The Fc region of the Ig molecule is involved in a wide variety of biological effector functions which include (1) control of catabolic rate, (2) C fixation, (3) binding to FcR, (4) anaphylaxis, (5) opsonization, (6) placental and gut transfer, and (7) immune regulation. To this latter point, the cellular molecular events involved in the activation of lymphocytes and macrophages by Fc fragments of Ig and the regulatory effects these fragments have on the immune response suggest a participation of the Fc region of antibody in IC-mediated immunoregulation. Since the cellular events in lymphocyte activation by Fc fragments and IC appear to be identical, perhaps immunoregulation by these agents occurs via similar pathways.

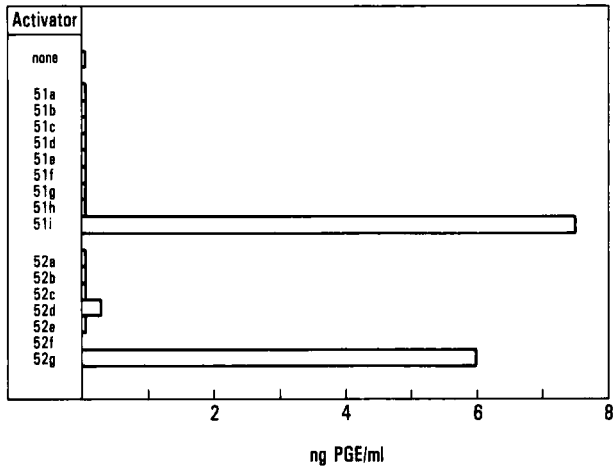

FIG. 30. Induction of PGE release from macrophage-enriched mouse spleen cells by synthetic p23 subpeptides. Cultures were stimulated with equimolar concentrations of peptides. Reprinted from Hobbs *et al.* (1986; see Table 21).

IC, aggregated human IgG_1, and Fc fragments cause proliferation and/or differentiation of mouse and/or human B cells. Fc fragments and their synthetic peptides were employed to dissect both the cellular and molecular events involved in B cell activation and the peptide sequences required for such activation. Fc fragments added to murine splenic or human PBM cultures results in proliferation of the B cells by subfragments generated from Fc fragments by cleavage with macrophage enzymes. These subfragments also stimulate T helper cells to release a soluble factor (BCAF) which causes the activated B cells to polyclonally secrete Ig. The ability of the Fc fragment to activate AA metabolic pathways may, in part, be involved in the regulation of both these events and the adjuvant effect of Fc fragments.

Chemical characterization of Fc fragments obtained from a human IgG_1 myeloma protein indicates that at least one biologically active site in the molecule is localized to a 23 amino acid region (residues 335–357) in the CH_3 domain of the molecule. Solid phase synthesis of the biological sequence resulted in a peptide, p23, which induces mouse and human B cells to secrete polyclonal Ig and activate AA metabolic pathways. In contrast to these results, Fc fragment-induced IL-1 release and thus B cell proliferation do not map to the p23 molecule. Analysis of data with overlapping peptides, based on the p23 sequence, suggests that the minimal active sequence needed for B cell differentiation is Leu_{351}-Pro_{352}-Pro_{353}-Ser_{354}-Arg_{355}. This peptide

TABLE XXII
BIOLOGICAL ACTIVITIES ASSOCIATED WITH THE Fc REGION OF Ig

Stimulator	B cell proliferation	Ig secretion	BCAF production	IL-1 production	Activation of AA cascade
Immune complexes	+	+	?	+	+
Aggregated γ-globulin	+	+	?	+	?
IgG_1	−	−	−	−	−
Fc fragments	+	+	+	+	+
Fab fragments	−	−	−	−	−
$F(ab')_2$ fragments	−	−	?	?	?
Pet-IgG_1 335–357	−	+	?	?	?
Synthetic peptides					
Eu IgG_1 (335–357)	−	+	+	−	+
Eu IgG_1 (335–355)	−	+	−	−	+
Eu IgG_1 (335–353)	−	+	+	−	−
Eu IgG_1 (351–357)	−	+	−	−	−

is localized to the carboxy terminus of p23. In contrast to these results, only intact p23 or p23 minus the carboxyterminal Glu_{356} and Glu_{357} were able to induce the release of PGE from monocytes. Taken together, these results indicate that the induction of polyclonal Ig secretion and release of PGE by mouse cells have different sequence requirements within the p23 molecule.

The release of biologically active peptides derived from the Fc fragment of antibody may be part of a potent nonspecific *in vivo* immunoregulatory network. In addition to the release of Fc region peptides, IC formation also results in C activation with the subsequent release of C fragments (C3a and C5a) that possess immunoregulatory properties. The interaction of these peptides and fragments with B cells, T cells, and macrophages in the cellular microenvironment could play a significant role in the control of immune responses.

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ADVANCES IN IMMUNOLOGY, VOL. 40

Immunoglobulin-Specific Suppressor T Cells

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I. Introduction

A milestone in immunology was the demonstration by Gershon and Kondo (1971) that some forms of tolerance were not simply due to a lack of reacting cells, but appeared to be mediated by active, specific, suppressive immune responses. They originally termed the effect "infectious tolerance" since it could be adoptively transferred with cells to a naive individual. The studies of Gershon and Kondo launched a period of intense experimentation that led to the identification of suppressor T cells and the development of numerous experimental systems in which such cells appeared to play important immunoregulatory roles. This subject has been reviewed (Green *et al.*, 1983; Dorf and Bernacerref, 1984; Herzenberg *et al.*, 1983).

Suppressor T cells have been a source of much controversy and to a large extent this has been fostered by (1) the indirect types of evidence from which the existence of suppressor T cells has been inferred, (2) the overwhelming complexity of most experimental systems in which suppressor T cells have been investigated, (3) the failure, thus far, to identify and fully characterize recognition and effector molecules that account for the observed specificity of suppressor T cell effects, (4) disagreement about the nature of the cellular targets of suppressor T cells, and (5) speculation that suppressor T cell effects reflect the activity of cytotoxic T cells.

Recent studies using murine plasmacytomas as monoclonal B cell models (Hanley-Hyde and Lynch, 1986) have begun to provide information that addresses some of these issues. One sequence of studies has established an experimental system in which (1) the target of the suppressor T cell is a B cell and not a helper T cell (Milburn and Lynch, 1982), (2) an idiotype : anti-idiotype interaction is the basis for recognition of the target B cell by the suppressor T cell (Lynch and Milburn, 1983), (3) the suppressor T cell inhibits the synthesis and secretion of immunoglobulin by the target B cell without influencing its growth or viability (Milburn and Lynch, 1982), and (4) the mechanism of suppression of immunoglobulin synthesis involves regulation

of expression and function of immunoglobulin messenger RNA (Parslow *et al.*, 1983; Milburn *et al.*, 1984).

It is becoming clear that suppressor T cells are heterogeneous in terms of their activities. The present article will focus on studies in which suppressor T cells appear to act directly on B cells. In some of these studies T cell recognition of an immunoglobulin epitope (e.g., idiotope, isotope, or allotope) triggers suppression, while in other studies T cell recognition of an epitope on a hapten–carrier conjugate captured by membrane Ig of the B cell triggers a suppressor effect. Systems in which suppressor T cells appear to be targeted to another T cell, such as a helper/inducer T cell, are well known and have been reviewed elsewhere (Green *et al.*, 1983; Dorf and Benacerraf, 1984; Herzenberg *et al.*, 1983; Asherson *et al.*, 1986).

II. Idiotype-Specific Inhibition of B Cells by Suppressor T Cells

BALB/c mice immunized with purified BALB/c myeloma protein develop antibodies (Sirisinha and Eisen, 1971; Odermatt *et al.*, 1978) and T cells (Rohrer *et al.*, 1979; Sakato *et al.*, 1982; Jorgensen and Hannestad, 1979) that recognize idiotypic antigens present on the monoclonal immunogen. Such mice resist challenge with otherwise lethal numbers of myeloma cells that express the corresponding idiotype (Lynch *et al.*, 1972). The mechanism of this idiotype-specific tumor immunity is undefined but a short-lived T cell appears to play an important role (Daley *et al.*, 1978).

Some mice that are idiotype immune develop tumors when they are overchallenged with a high dose of myeloma cells. Interestingly, the tumors that develop in these mice exhibit marked alterations in immunoglobulin expression compared to the original cells used to challenge (Lynch *et al.*, 1972). In some immunized mice tumors developed but a serum monoclonal protein did not. When cells from such a tumor were transferred from the immune host to a nonimmune recipient the tumors that developed were once again accompanied by high serum concentrations of the monoclonal protein. These observations were clarified in later experiments in which idiotype-immune mice were implanted with peritoneal diffusion chambers containing myeloma cells (Rohrer *et al.*, 1979). The myeloma cells used were from MOPC-315, a clone that produces M315, an IgA λ_2 anti-TNP antibody (Eisen *et al.*, 1968). The mechanism that mediates tumor immunity does not operate when MOPC-315 cells are enclosed in 0.1-μm-pore diffusion chambers, implying that cell : cell contact is required to mediate what appears to be a cytostatic effect. Thus the

growth of MOPC-315 cells in diffusion chambers was comparable in idiotype-immune and control mice (Rohrer *et al.*, 1979). In contrast, little or no M315 could be detected in the serum of idiotype-immune mice implanted with diffusion chambers containing MOPC-315 cells, while high M315 titers were found in the sera of control or normal mice. MOPC-315 cells recovered from diffusion chambers of normal mice showed a high frequency of plaque formation on TNP-SRBC, while MOPC-315 cells recovered from idiotype-immune mice failed to form plaques (Rohrer *et al.*, 1979). The inhibition of M315 secretion was shown to be idiotype specific, reversible, and mediated by T cells that acted directly on the MOPC-315 cells. Id^{315}-specific suppression of M315 secretion could be adoptively transferred to normal mice with spleen cells from M315-immunized donors and the transfer could be abrogated by treatment of the immune spleen cells with anti-Thy-1 antibody plus complement. Furthermore, in contrast to actively immunized mice, adoptively immunized mice neither developed anti-Id^{315} antibodies nor suppressed surface IgA^{315} expression. These findings established that idiotype-specific T cells could inhibit M315 secretion and provided strong evidence that myeloma cells might be useful clonal systems for the study of some aspects of B cell regulation. The suppression was shown to be idiotype-specific since T cells from mice immunized with M460, an IgAk anti-TNP myeloma protein, failed to inhibit M315 secretion but did inhibit M460 secretion by MOPC-460 cells. An interesting finding was the rapid reversal of secretory inhibition following brief exposure of suppressed MOPC-315 cells to Pronase (Rohrer *et al.*, 1979). This finding was one piece of evidence that suggested suppression was mediated at the level of the actual antibody-secreting cell. Since suppression was achieved across the 0.1-μm pores of diffusion chamber membranes the effect appeared to be mediated by a soluble factor and it did not require T cell : B cell contact.

That these findings might be relevant to the regulation of normal B cells was suggested by earlier studies (Baker, 1975; Basten *et al.*, 1975; Swain, 1978), in which suppressor T cells appeared to act directly upon the actual antibody-secreting cell. Additional evidence for this site of action came from studies reported by Warren and Davie (1977), in which immunogen bound to the surface of antibody-secreting cells served as a target for suppressor T cells (or products) that resulted in a striking inhibition of antibody secretion. In a study reported by Watanabe *et al.* (1978) a T hybrid cell line produced an IgE class-specific suppressor factor that acted directly on B cells to inhibit IgE production. On the basis of these and later studies discussed

below it has become clear that the previous widely held belief that suppressor T cells inhibited antibody responses solely by inhibition of helper T cells is incorrect.

The great advantage that comes from studies with cloned B cell tumors is the opportunity it provides for a detailed molecular analysis of some of the mechanisms involved in immune regulation. As discussed above and reviewed in detail elsewhere (Hanley-Hyde and Lynch, 1986; Abbas, 1982) the patterns of responses of B cell tumors to regulatory signals often mimic patterns seen in studies that use normal B cells. Moreover, information developed from tumor cell models of B cell function has sometimes provided additional insights and has rarely, if ever, been misleading. The aberrant features expressed by B cell tumors often turn out to be features expressed by normal B cells but which had simply evaded discovery. A good example is the "aberrant" expression of the putative T cell marker Ly1 on B lymphoma cells (Lanier *et al.*, 1981) that preceded its detection as a marker of an important subset of normal B cells (Hayakawa *et al.*, 1983). In some instances neoplastic B cells express what truly appear to be aberrant features such as the production of truncated immunoglobulin chains. Even here, however, analyses of the chemical structures of the truncated chains have been illuminating and actually predicted the existence of intervening DNA sequences in immunoglobulin heavy chain genes (Seligmann *et al.*, 1979) prior to the development of the recombinant DNA technology that formally established this point (Hozumi and Tonegawa, 1976).

The finding that idiotype-immune mice suppressed the secretion of the corresponding idiotype by myeloma cells was initially a surprising result and one that was difficult to explain. Since idiotype-immune mice developed anti-idiotypic antibodies (Lynch *et al.*, 1972) it was possible that these were involved but it was difficult to imagine how they could encounter myeloma cells because the secreted idiotype would bind the anti-idiotypic antibodies and neutralize them. A rather extensive set of studies failed to develop any evidence for anti-idiotypic antibodies by themselves playing a direct role in suppression of secretion (Frikke *et al.*, 1977; Milburn and Lynch, 1983). Thus, high concentrations of syngenic, polyclonal anti-Id^{315} antibodies or IgG_1 anti-Id^{315} monoclonal antibodies failed to influence secretion of M315 *in vitro* when continously present in the culture for as long as 3 weeks. However, it is known from a number of studies in other laboratories that administration of an anti-immunoglobulin [e.g., anti-idiotype (Rajewsky and Takemori, 1983), anti-allotype (Herzenberg and Herzenberg, 1974)] can lead to a state of specific suppression

which, once established, can be transferred with T cells. In the MOPC-315 model idiotype-specific suppression of secretion can be transferred to naive mice with immune T cells in the absence of anti-idiotypic antibodies (Rohrer *et al.*, 1979). However, since the idiotype-specific suppressor T cells developed in immunized mice that had also developed anti-idiotypic antibodies it is possible that the development of the suppressor T cells requires anti-idiotypic antibodies. A principle that appears to operate in some models of idiotype network regulation is that access to the regulatory circuits can be achieved either by immunization with idiotype or treatment with anti-idiotypic antibodies (Rajewsky and Takemori, 1983). In either case T cell-mediated effects are induced. Important points that need to be addressed are the importance of the anti-idiotypic heavy chain class or subclass, and whether Fc receptors participate in the development of these suppressor circuits.

Milburn and Lynch (1982) developed an *in vitro* system that provided a means to further dissect the mechanisms by which idiotype-specific suppressor T cells inhibited the secretion of immunoglobulin by murine myeloma cells. When nylon wool nonadherent spleen cells from mice immunized with M315 were cocultured with a long-passage cloned line of MOPC-315 cells there was no effect on the growth or viability of MOPC-315 cells, or on the surface membrane expression of M315. However, there was a striking decrease in secretion of M315 which was dependent on the dose of immune spleen cells used. At high effector : MOPC-315 cell ratios there was virtually total suppression of M315 secretion. When the immune spleen cells were depleted of $Ly2^+$ cells the suppressive effect was totally abrogated. Depletion of the $Ly1^+$ cells did not influence the suppressive effect. When equal numbers of Ly1-depleted and Ly2-depleted cells were mixed and added to MOPC-315 cultures, the level of suppression observed was the same as with the same number of Ly1-depleted cells alone. This suggested that (1) $Ly1^+$ cells do not augment or inhibit the $Ly2^+$ effectors, and (2) the $Ly1^+2^+$ cells, which were depleted by passage through either column in these mixing experiments, were not the effectors and were not required at the effector stage of M315 secretory inhibition. These studies also established that accessory cells were not required at the effector stage of suppression. Solid phase idiotype columns established that the immune spleen T cells had surface membrane structures that recognized and bound to M315 but not to M460, an IgAk anti-TNP myeloma protein that expresses a dissimilar idiotype (Milburn and Lynch, 1982). T cells eluted from Id^{315} columns were enriched for specific suppressor cells. The nature

of the idiotype recognition structure on the T cells has not been determined.

Inhibition of M315 secretion was detected as soon as 6 hours after initiating cocultures of immune spleen T cells and MOPC-315 cells (Milburn and Lynch, 1982). Since the target cells were secreting M315 at the time the suppressor T cells were added to the culture, it is clear that the suppressor T cells act directly on the actual antibody-secreting cell rather than on a precursor cell. It seemed unlikely that inhibition of M315 secretion resulted from impaired release in the presence of unchanged M315 synthesis because suppressed MOPC-315 cells were normal by ultrastructural analysis yet when M315 release, but not synthesis, was blocked by tunicamycin this was accompanied by a striking dilatation of endoplasmic cisternae filled with M315 (Hickman *et al.*, 1977).

When M315 synthesis was measured in suppressed MOPC-315 cells a greater than 90% inhibition was observed in the presence of Id^{315}-specific suppressor T cells (Milburn and Lynch, 1982). Since the suppressor effect was mediated by a diffusable product it was possible to examine total as well as specific protein synthesis in MOPC-315 cells by carrying out the experiments in a modified Marbrook chamber. [^{3}H]Leucine incorporation into intracellular M315 and the TCA-precipitable fraction of MOPC-315 cells showed that the inhibition of protein synthesis was selective for M315. Thus the entire decrement in total protein synthesis in MOPC-315 cells in the presence of the suppressor effect could be accounted for by the decrement in specific M315 synthesis. These findings suggested that inhibition of M315 secretion by idiotype-specific suppressor T cells was achieved by a selective down regulation of M315 synthesis.

Subsequent studies by Parslow *et al.* (1983) and by Milburn *et al.* (1984) provided insight into the molecular mechanisms that underly the inhibition of M315 synthesis by MOPC-315 cells in response to idiotype-specific suppressor T cells. When poly(A) RNA from nonsuppressed MOPC-315 cells was analyzed by Northern Blot with a λ_2 constant region probe, autoradiography revealed a single λ_2 transcript 1.2 kb in length representing the mature λ_2 mRNA, but this transcript was absent or markedly reduced in the RNA obtained from suppressed MOPC-315 cells (Parslow *et al.*, 1983). The same result was obtained whether the idiotype-immune suppressor T cells were cocultured directly with, or membrane segregated from, the MOPC-315 cells.

An additional interesting finding was made when the same RNA preparations were analyzed with a hybridization probe specific for the

λ_1 constant region. It was found that the expression of the λ_1 transcript precisely paralleled that of the λ_2 transcript, suggesting a coordinated regulation of the productively rearranged λ_2 and the aberrantly rearranged λ_2 genes in MOPC-315 cells. It had been previously shown that the aberrantly rearranged λ_1 gene in MOPC-315 cells is expressed and the truncated transcript (1.1 kb) produced codes for a peptide that is synthesized in small amounts and contains the λ_1 constant region but is devoid of most of the λ_1 variable region (Bothwell *et al.*, 1981; Schwartz *et al.*, 1981). Since the rearranged λ_2 and λ_1 genes in MOPC-315 cells reside on different chromosomes (Hozumi *et al.*, 1982) their coordinate regulation occurs via a *trans* mechanism. In this regard it may be significant that λ_1 and λ_2 constant region genes show a considerably higher degree of nucleotide sequence homology to each other than to the κ locus, reflecting a close evolutionary relationship between the λ genes (Selsing *et al.*, 1982). The variable region coding elements of λ_1 and λ_2 also exhibit substantial sequence homology: this homology extends several kilobases upstream from the coding regions themselves (Bothwell *et al.*, 1981; Schwartz *et al.*, 1981). Coordinate regulation of λ_1 and λ_2 gene expression in MOPC-315 may reflect the presence of conserved regulatory sequences common to both genes.

In contrast to the striking changes in light chain messenger RNA expression, the steady-state concentration of α heavy chain messenger RNA expression in MOPC-315 cells was not significantly altered by suppressor T cell action, implying that the inhibition of light chain messenger RNA expression is relatively selective and is not merely a result of nonspecific RNA degradation (Milburn *et al.*, 1984). Further evidence in support of this is the finding that J chain messenger RNA expression is not altered by suppressor T cell action (G. Lanmon, G. Milburn, M. Koshland, and R. Lynch, unpublished observations). In addition to normal steady-state concentrations of secretory and membrane forms of the α chain messenger RNAs in suppressed MOPC-315 cells, the α messenger RNA isolated from suppressed cells was translated *in vitro* in a cell-free translation system (Milburn *et al.*, 1984). This suggests that α-messenger RNA in suppressed MOPC-315 cells is a functionally intact molecule and it establishes a linkage between inhibition of light chain messenger RNA expression and post-transcriptional inhibition of heavy chain expression. Overall, these findings shed some light upon the control of immunoglobulin expression in B cells that have been influenced by specific suppressor T cells, and they identify an important regulatory role for the light chain in controlling the expression of the heavy chain (reviewed in Milburn *et al.*, 1984).

The initial encounter between the B cell and the appropriate suppressor T cell is highly specific and relies on recognition of the idiotype. It was shown that the suppressor T cell recognizes an idiotope located in the variable region of the α heavy chain (Lynch and Milburn, 1983). This assignment is particularly interesting because idiotype-specific helper T cells induced by immunization with M315 recognize an idiotope that appears to be located in the third hypervariable region of the light chain (Bogen *et al.*, 1985), while the anti-idiotypic antibodies that are induced are specific for combinatorial (V_L–V_H) idiotopes (Odermatt *et al.*, 1978). These findings demonstrate the interesting association of distinct functional effectors with the recognition of different idiotopes of the same immunoglobulin molecule. The significance of this pattern will likely remain unclear until other idiotype : anti-idiotype systems are studied at this level. Further studies are needed to determine whether changes in messenger RNA and heavy and light chain expression in MOPC-315 cells occur in response to other immunoregulatory signals, and to determine whether these mechanisms also operate in other B cells that come under immunoglobulin-suppressing influences of regulatory T cells. It is of considerable interest that disulfide-linked light chains appear to be regulatory subunits of two other heterodimeric membrane glycoproteins, class I MHC molecules (Maloy and Coligan, 1985) and leukocyte adhesion factors (LAF) (Springer *et al.*, 1984). As in the case of immunoglobulin, MHC and LAF are surface membrane recognition molecules composed of an integral membrane heavy chain and an associated, nonmembrane anchored light chain that appears to be a regulatory subunit in the process of molecular assembly.

In an interesting series of experiments Abbas and colleagues have investigated suppression of M315 secretion by idiotype-specific suppressor T cells (Abbas *et al.*, 1980, 1982; Moser *et al.*, 1985). The suppressor T cells are induced by intravenous immunization of BALB/c mice with M315-coupled BALB/c splenocytes. The idiotype-immune T cells elaborate a suppressor factor (TsF) that inhibits M315 secretion only in the presence of Ia^+ accessory cells (AC) and is idiotype specific. Suppression does not occur when myeloma targets and AC are separated by cell impermeable membranes, suggesting that the role of the AC may be to bind, focus, and/or present the TsF to MOPC-315 cells. This experimental system differs in several important details from the studies of Milburn and Lynch (1982), but has in common a specific suppressor T cell that triggers an effect that operates on a B cell target rather than on a helper T cell. An advantage of B cell tumor models is that the B cell does not depend on specific helper

T cells for activation and clonal expansion but instead the cells exhibit features that are often designated as autonomous. This property creates an experimental system in which it is possible to dissect suppressor T cell effects that operate in the absence of helper T cells.

III. Antigen-Specific Inhibition of MOPC-315 by Suppressor T Cells

A sequence of studies (Rohrer and Lynch, 1978, 1979) demonstrated that antigen-specific suppressor T cells could inhibit proliferation, differentiation, and M315 secretion by MOPC-315 cells *in vivo*. In those studies suppressor T cells specific for sheep erythrocytes (SRBC) inhibited MOPC-315 cells contained in peritoneal diffusion chambers that also contained TNP-SRBC and AC. The suppressor effect required the presence of AC in the chamber but the suppressor T cells were able to function across the 0.1-μm-pore membrane. This suggested that the role of the AC was to bind, focus, and/or present the TNP-SRBC antigen and/or a suppressor factor elaborated by the T cells. The suppressor effect was antigen specific and exhibited the restriction of linked recognition described earlier by Mitchison (1971) for regulation of normal B cells. Thus when the TNP was present on mouse erythrocytes even in the presence of nonhaptenated SRBC suppression did not occur; there was a strict requirement for the TNP to be present on the same erythrocyte species that was used to induce the suppressor T cells. In summary, the observations of Rohrer and Lynch (1978, 1979) suggested that a carrier-specific TsF interacted with an accessory cell : carrier–hapten complex and this was bound to the target MOPC-315 cells by the TNP-binding sites of the surface membrane M315. Those studies established the specificity of the triggering events that led to suppression; they did not establish the specificity of the actual effector molecule.

In recent studies G. Moser *et al.* (personal communication) observed that TNP-specific TsF inhibits M315 secretion in the presence of TNP-protein and Ia^- AC. This form of suppression is TNP specific at the triggering stage but is nonspecific at the effector stage since a non-TNP-binding myeloma cell, TEPC-15, is suppressed as long as TNP–protein and activated macrophages are present. This form of suppression occurs even when the myeloma targets and AC are separated by 0.2-μm-pore membranes.

Overall, the studies of Milburn and Lynch (1982), Rohrer and Lynch (1978), Abbas *et al.* (1980, 1982), and Moser *et al.* (1985) have identified distinct mechanisms by which suppressor T cells inhibit M315 secretion by MOPC-315 cells. So far data are available only for

the biochemical changes that occur in MOPC-315 cells when they are suppressed by an idiotype-specific TsF that functions in the absence of AC (Milburn and Lynch, 1982). As discussed above, this suppression involves inhibition of light chain mRNA expression (Parslow *et al.*, 1983). It will be interesting to see if the other suppressor systems discussed trigger different biochemical changes that lead to inhibition of secretion, or whether a final common pathway is involved in the target B cell.

IV. T Cell-Mediated Suppression of Malignant B Cell Proliferation

In addition to suppressor T cells that appear to act on B cells late in differentiation to inhibit immunoglobulin expression, a number of studies have established that specific T cells can act on B cells to suppress proliferation and clonal expansion. Virtually all of these studies have used experimental systems in which the target B cell was a tumor cell. Thus syngenic BALB/c mice immunized with purified monoclonal immunoglobulins are rendered specifically resistent to challenge with otherwise lethal numbers of tumor cells that produce that idiotype (Lynch *et al.*, 1972; Meinke *et al.*, 1974; Eisen *et al.*, 1975). In a different system idiotype-specific tumor protection against an anti-arsonate hybridoma clone was adoptively transferred with immune T cells from idiotypically suppressed mice (Kresina *et al.*, 1983). While the mechanisms that mediate tumor immunity in these systems are yet to be fully understood, there is some evidence that favors a cytostatic effect, and so far, no evidence for a cytotoxic effect. Thus, idiotype-immune T cells are not cytotoxic *in vitro* (Milburn and Lynch, 1983) and the tumor immunity is ablated by postimmunization thymectomy (Daley *et al.*, 1978), suggesting a short-lived regulatory effector rather than a conventional cytotoxic T cell. Moreover, preliminary observations indicate that MOPC-315 cells are not immediately destroyed in immune mice but persist for variable periods of time. While clearly not definitive, this type of evidence raises the possibility that the T cell-mediated protection may be a cytostatic effect rather than a cytotoxic effect. In a series of studies (Rohrer and Lynch, 1978, 1979) it was established that carrier-specific suppressor T cells inhibited the proliferation of TNP-specific MOPC-315 plasmacytoma cells *in vivo*. The suppressor effect was mediated by a soluble product of the T cell, required AC in contact with MOPC-315 cells, and exhibited the restriction of linked recognition originally described for the regulation of normal B cells (Mitchison, 1971).

In summary, idiotype-specific and antigen-specific suppressor T cells appear to have at least two distinct types of mechanisms by which they suppress B cell function: (1) inhibition of immunoglobulin expression and (2) inhibition of proliferation and clonal expansion.

V. Isotype-Specific Inhibition of B Cells by Suppressor T Cells

Evidence has existed for some time that T cells may specifically influence the immunoglobulin class expressed during immune responses. Studies of IgE, IgA, and IgG subclass expression have provided evidence in favor of this view. In addition, alterations in immunoglobulin class expression in certain diseases has also suggested that specific T cells might be involved.

In principle, regulation of immunoglobulin class expression by T cells could involve isotype-specific helper T cells, T cells that influence heavy chain class switching, and isotype-specific suppressor T cells. Evidence has been presented in support of each of these mechanisms but the present article will only consider those studies that have provided evidence for the existence of isotype-specific suppressor T cells.

An early hint that T cells might be involved in the regulation of immunoglobulin class expression was the finding that FcR^+ T cells released an immunoglobulin-binding factor (IBF). This was originally reported by Fridman and Golstein (1974) for normal T cells and by Fridman *et al.* (1977) for T lymphoma cells. Neuport-Sautes *et al.* (1975, 1979) showed the relationship between the specificity of the T cell Fc receptor and the shed IBF. Unkeless (1979) constructed a monoclonal antibody (2.4G2) directed against murine macrophage and lymphocyte Fc receptors and Daeron *et al.* (1984) found that $Fc\gamma R$ and IgG-BF expressed common antigenic sites recognized by the 2.4G2 antibody. Lowy *et al.* (1983) observed that a murine T cell hybridoma (T2D4) could be selectively induced to produce IgG-subclass specific IBFs that exerted a suppressive effect on antibody reaction by murine spleen cells. The chemical structure of γ-IBF from murine T cells is partially known and a recent review (Neuport-Sautes *et al.*, 1986) addressed the status of those studies.

Studies in normal humans (Moretta *et al.*, 1978) identified $Fc\gamma R$ and $Fc\mu R$ on distinct populations of peripheral blood T lymphocytes. In a pokeweed-driven *in vitro* system of Ig secretion by human PBL the $Fc\gamma R$ T cells exhibited a suppressive effect while the $Fc\mu R$ T cells exhibited a helper effect. Subsequent studies have confirmed an immunoregulatory function for human FcR^+ T cells but have cast doubt

on the validity of a strict assignment to exclusive helper or suppressor categories based on the class of Fc receptor expressed. The specificity and the mechanisms by which human FcR^+ T cells mediate a suppressive effect on Ig secretion remain unsettled. Nonetheless, the identification of FcR^+ T cells in humans, the demonstration that a component of these cells can mediate a suppressive effect on B cell function, and the finding of altered FcR^+ T cells in some disease states are each responsible for an increased interest in human FcR^+ T cells.

An important development has been the finding in experimental animals and humans that cross-linking forms of immunoglobulin upregulate the expression of Fc receptors on lymphoid cells. Whether this is due to an increased number, density, or avidity of receptors is an issue under investigation in a number of laboratories. Exposure of murine T cells to high concentrations of immunoglobulin results in an increased expression of FcR specific for the heavy chain class of the inducing immunoglobulin. Yodoi *et al.* (1979) observed increased $Fc\varepsilon R$ expression on rat and murine lymphocytes exposed to cross-linking forms of IgE *in vitro*. In a sequence of studies in mice bearing Ig-secreting plasmacytomas and hybridomas increased numbers of $L3T4^-$, $Ly2^+$ T cells were found to express surface receptors whose specificity matched the heavy chain class of the monoclonal protein secreted by the tumor. This has been established for IgA (Hoover and Lynch, 1980) IgG (Mathur and Lynch, 1986), IgM (Mathur and Lynch, 1986), and IgE (Mathur *et al.*, 1986).

Gebel *et al.* (1979) and Hoover and Lynch (1980) observed elevated numbers of $Ly2^+$ $Fc\alpha R^+$ cells in mice with IgA-secreting plasmacytomas but not in mice with tumors of variant clones that did not secrete IgA. It was subsequently shown that normal mice infused with polymeric IgA developed increased numbers of $Fc\alpha R^+$ T cells and that normal splenic T cells cultured in the presence of polymeric IgA developed increased expression of $Fc\alpha R$ (Hoover *et al.*, 1981a). By cell surface marker analysis the increased $Fc\alpha R$ T cells were phenotypically suppressor T cells and were not found in adult-thymectomized or nude mice bearing IgA-secreting plasmacytomas (Hoover *et al.*, 1982). When $Fc\alpha R$-enriched spleen cells were transferred into normal mice it resulted in almost complete suppression of the IgA component of an established immune response without influencing the IgG or IgM components (Hoover and Lynch, 1983). The rapidity of the suppressor effect suggested that it was mediated at the level of the IgA-producing B cell.

Studies by Mathur and Lynch (1986) established that mice bearing IgG-secreting plasmacytomas or hybridomas developed increased

numbers of L3T4$^-$, Ly2$^+$ T cells that expressed FcγR. Nonsecretory variant tumors did not induce changes in FcγR T cells. Furthermore, mice bearing IgM-secreting plasmacytomas or hybridomas developed large number of T cells that expressed surface FcμR but FcμR T cells were not increased in mice with tumors of nonsecretory variants (Mathur and Lynch, 1986). In each of these studies the enhanced expression of FcR was specific for the heavy chain class of the inducing immunoglobulin. Mice with IgG-secreting tumors did not develop increased expression of FcμR or FcαR on T cells. Likewise, mice with IgM-secreting tumors did not develop increased expression of FcαR or FcγR on T cells. Comparable findings have been made in humans with multiple myeloma (Hoover *et al.*, 1981b).

Recent studies by Mathur *et al.* (1986) have established that mice bearing an IgE-secreting hybridoma developed a marked increased in L3T4$^-$, Ly2$^+$ T cells that express FcεR. These did not develop in mice with nonsecreting variant tumors but did develop in mice infused with large concentrations of IgE. Interestingly, the development of FcεR T cells was followed by decreased serum levels of the monoclonal IgE and a decrease in IgE production by the hybridoma cells (unpublished observations). T cell-mediated suppression of IgE secretion by hybridoma cells grown in Ig-allotype congenic mice has been reported by Maekawa *et al.* (1986).

In a series of studies Ishizaka and colleagues (1984) have developed evidence for T cells that specifically inhibit IgE synthesis by B cells. They have proposed that the state of glycosylation of an IgE-specific IBF produced by a T cell determines whether the IBF suppresses or enhances IgE expression. The IgE-binding factor presumably binds to membrane IgE on the B cell as a step in effecting the suppression. Earlier studies by Suemura *et al.* (1983) identified an IgE-specific suppressor factor produced by a T cell hybridoma that inhibited IgE production by a B hybridoma.

Based on the studies reviewed above some conclusions can be drawn and some inferences made. It is now reasonably well established that in experimental animals and humans T cells exist that have the phenotypic markers of suppressor T cells and that express isotype-specific Fc receptors on their surface. In most cases studied a released IBF whose specificity corresponds to the FcR can be found. It has been shown that cross-linking forms of immunoglobulin can upregulate the expression of the corresponding Fc receptor on suppressor T cells *in vitro* and *in vivo*. This has been shown for IgA, IgE, IgM, and IgG. The upregulated FcR is specific for the heavy chain class of the inducing immunoglobulin. In some of the studies reviewed there is

evidence suggesting that the $Ly2^+$ FcR T cell participates in an isotype-specific suppressor effect that is mediated at the level of a B cell target.

It can be inferred from these studies that the elements of isotype-specific suppressor circuits exist and that B cells, via the immunoglobulin molecules they secrete, are not only effector cells but are also important immunoregulatory cells. It needs to be emphasized that FcR are expressed on other subsets of T cells, as well as on many other lymphoid and nonlymphoid cells, and cannot in any way be considered a marker of suppressor T cells. Furthermore, while the influence of immunoglobulin on FcR expression has been emphasized here, a multiplicity of agents such as interferons, lymphokines, lymphocyte activators, and divalent cations profoundly influence FcR expression (Neuport-Sautes *et al.*, 1986). Because of this it is not difficult to imagine how alterations in FcR^+ T cell expression might occur in response to a pathogen or following tissue injury and lead to a pathologic expression of T suppressor activity. It is interesting in this regard that FcR^+ T cell alterations have been reported in a number of diseases that have elements of impaired B cell function. These include multiple myeloma (Hoover *et al.*, 1981b), hyper-IgE syndrome (Dreskin *et al.*, 1985), selective IgA deficiency, and IgA nephropathy (Adachi, 1983).

VI. Summary

This article has considered evidence that supports the occurrence and functional importance of suppressor T cells that are directed to B cell targets. Cells with these features have been demonstrated in experimental animals and in humans. The designation "suppressor" comes from the serologic phenotype of these cells as well as from their functional property of noncytotoxic inhibition of B cell function.

Distinct suppressor T cells with these properties have been identified that effect antigen-, idiotype-, isotype-, and allotype-specific suppression of B cell function. While such cells had been suspected from earlier studies of normal immune responses, the development of monoclonal B cell models using tumor cells has provided a means to readily detect these suppressor T cells and to investigate the mechanisms by which they mediate their effects. Tumor models have proved to be powerful tools in the effort to identify and analyze the elements that underlie the complexity of immune responses. Combined with the insights provided by molecular genetic approaches and flow cytometry, functional and responsive lymphoid tumor cells

are being used with increasing frequency to address basic immunoregulatory issues.

An important family of suppressor T cells with B cell targets are those that express surface Fc receptors, elaborate immunoglobulin-binding factors, and appear to participate in the regulation of immunoglobulin heavy chain class expression. In addition to their importance in the regulation of heavy chain class expression during normal immune responses, alterations in FcR^+ T cells in a number of disease states may provide clues that will lead to a better understanding of disorders of immune regulation.

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Immunoglobulin A (IgA): Molecular and Cellular Interactions Involved in IgA Biosynthesis and Immune Response

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I. Introduction

A well established finding that in many species, including humans, the daily production of IgA exceeds production of immunoglobulins of all other isotypes is infrequently acknowledged in the immunological literature (Heremans, 1974; Delacroix, 1985; Mestecky *et al.*, 1986b). This may be explained by the lower levels of serum IgA relative to IgG which, however, do not consider the unique biosynthesis, body fluid distribution, and catabolism of IgA. In addition to serum, large amounts of IgA are also found in external secretions including tears, saliva, colostrum, and milk, and respiratory, gastrointestinal, and genitourinary secretions (for review see Heremans, 1974). In some species the systemic and secretory compartments display a considerable degree of independence. For example, in man the majority of serum IgA is primarily produced in the bone marrow and does not enter external secretions in significant amounts while IgA found in most of the secretions is locally produced by plasma cells characteristically distributed in secretory tissues but does not contribute significantly to the circulatory pool (Heremans, 1974; Delacroix, 1985; Mestecky *et al.*, 1986a). Recent results (Delacroix, 1985) indicate that the combined synthesis of systemic and secretory IgA (S-IgA) (approximately 66 mg/kg/day) inevitably places IgA as the predominant immunoglobulin produced in humans. A need for the synthesis of such large quantities of IgA may be well justified. Mucosal surfaces represent the largest area of contact of the immune system with the environment. Microorganisms and their products and substances present in ingested food and inhaled air provide a constant and potent source of stimulation with foreign antigens for both secretory and systemic immune compartments. It can be safely concluded that most of the immunological experiments performed in animal models and in humans are carried out on the background of exposure to environmental antigens that profoundly influence the outcome of systemic immunization. Therefore, it is not surprising that the intestines contain the

largest accumulation of lymphoid tissues in the body (Brandtzaeg, 1985). This fact is often underappreciated and the cellular responses measured in spleen and lymph nodes, organs favored by most immunologists, may not reflect the entire immune response induced by either systemic or mucosal immunizations.

From the phylogenetic point of view IgA has been considered as a relative newcomer. It has been reliably detected by immunochemical means in birds (Vaerman, 1973) but recent studies based on antigenic analyses of so-called IgY immunoglobulins from lower vertebrate species (birds, reptiles, and amphibians) suggest that these molecules display a close antigenic relationship to mammalian IgA (Hadge and Ambrosius, 1984). Accordingly, it appears that the heavy (H) chains of IgA (α chains) had evolved from the μ chain 350 million years ago, before the evolution of the γ chain. Further sequence studies of α chains or DNA sequences will be required to confirm these conclusions. The purpose of this article is not to provide a comprehensive summary of the structural, functional, and cellular aspects of IgA so exquisitely assembled by Heremans (1974) but to reconsider and assess the impact of new information concerning IgA physiology on the immune system. We hope that the reader will realize that IgA should not be considered only as an isotype providing specific humoral protection of mucosal surfaces but as an integral component of the entire immune system.

II. Structure and Function of Component Polypeptide Chains of Serum and Secretory IgA

An unusual structural feature of human IgA is the heterogeneity of the molecular forms with characteristic distribution in various body fluids. Though most IgA in serum displays a typical four-polypeptide chain structure of the basic molecule with two α and two light (L) chains, external secretions contain dimeric and tetrameric, disulfide-linked molecules associated with additional polypeptides—J (joining) chain and secretory component (SC) (for review see Heremans, 1974). The latter glycoprotein is derived from various epithelial cells and the assembled molecule of S-IgA is a product of two entirely different cell types: plasma cells that produce IgA with J chain and epithelial cells that produce SC. Yet, despite the differences in the cellular origins, IgA with J chain, and SC exhibit similar domain structures (see below). The primary structure of all component chains of serum and S-IgA has been determined in several laboratories. Information concerning human α chains from paraproteins of both subclasses is

available in Kratzin *et al.* (1978), Putnam *et al.* (1979), Torano and Putnam (1978), Tsuzukida *et al.* (1979), mouse α chains in Robinson and Appella (1980), Auffray *et al.* (1981), and Tucker *et al.* (1981), human and mouse J chain in Mole *et al.* (1977), Max and Korsmeyer (1985), Cann *et al.* (1982), and rabbit and human SC in Mostov *et al.* (1984) and Eiffert *et al.* (1984).

A. Structure of α Chains

A comparative schematic diagram of the constant regions of human α chains of IgA_1 and IgA_2 of both allotypes is shown in Fig. 1. The constant region is composed of 3 domains with relatively minor differences in the primary structure: only 22 of 365 amino acids differ in the entire C region of $\alpha 1$ and $\alpha 2$ chains. The C terminus of α chain is similar to that of μ chain and extends over C termini of γ, ε, and δ chains (Lin and Putnam, 1981) (Fig. 2). In polymeric IgA the penultimate cysteine residue forms a disulfide bridge with cysteine residues of J chain (Mendez *et al.*, 1973; Mestecky *et al.*, 1974a). In the monomers penultimate cysteine residue forms a disulfide bond with an unidentified cysteine residue located closer to the N terminus of the same α chain or the other α chain of the monomeric subunit as proposed by Prahl *et al.* (1971). Yang *et al.* (1979) have shown that the penultimate cysteine forms a disulfide bridge with the homologous cysteine on the other α chain. The extensions seen in both α and μ chains strongly suggest that this part of the molecule is involved in the ability of immunoglobulins of IgA and IgM isotypes to form polymers.

The precise location of intra- and intersubunit disulfide bridges in the α chain is not completely resolved. A total of 17 cysteine residues in the α chain makes this task difficult. Monomeric subunits within polymeric IgA appear to be linked by disulfide bridges found in three locations in the Fc region: (1) penultimate cysteine residues form a disulfide bridge directly connecting two monomers, (2) penultimate cysteine residues are linked to J chain which in turn joins two monomers, and (3) an additional disulfide formed between cysteine residues 311 (Chapuis and Koshland, 1975; Mestecky, 1976; Yang *et al.*, 1979; Garcia-Pardo *et al.*, 1981). These considerations are summarized in Fig. 3. However, there are several unresolved issues that will require further studies. First, the number of J chain molecules per polymeric IgA appears to differ in various myeloma proteins (Brandtzaeg, 1975a, 1976a). Furthermore, it has not been conclusively determined whether J chain indeed connects two monomeric subunits or whether it is associated with one of the subunits. Studies of Garcia-Pardo *et al.* (1981) are open to alternative explanations (Mestecky,

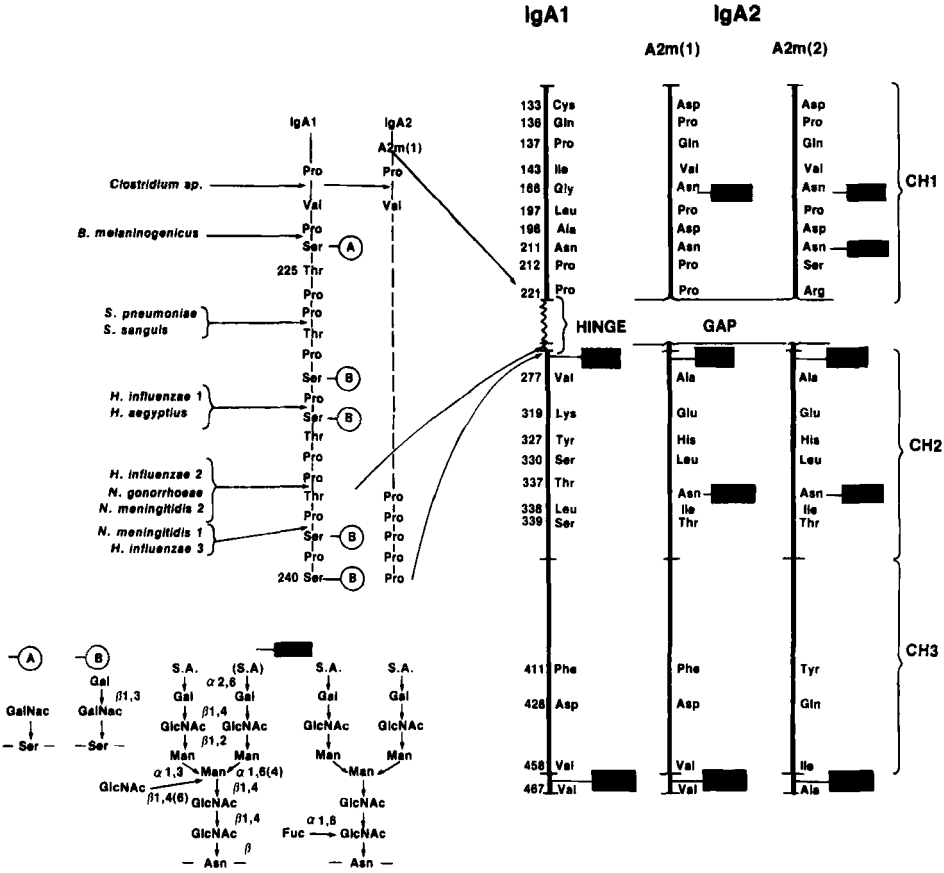

FIG. 1. Structural differences in the constant region domains CH1, CH2, and CH3 of human myeloma proteins of IgA_1 and IgA_2 isotypes, including A2m(1) and A2m(2) allotypes; numbers indicate positions of amino acid residues in the $\alpha 1$ chain (myeloma protein Bur., Putnam *et al.*, 1979) and corresponding substitutions in the $\alpha 2$ chains. The HINGE region in the $\alpha 1$ chain is expanded on the left and sites of cleavages with IgA_1 proteases produced by different bacterial species are indicated by arrows. GAP indicates a deletion in $\alpha 2$ chains. Structures of N-glycosidically (Asp, ■) and O-glycosidically (Ser, ○) linked oligosaccharide side chains in constant region domains and in the hinge region are shown (bottom left).

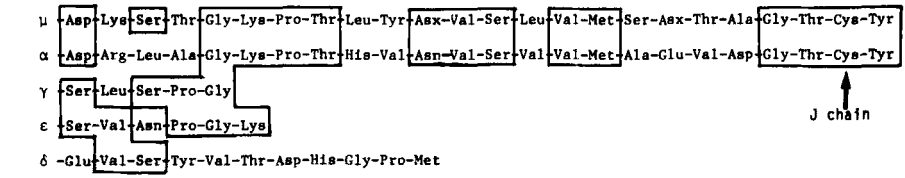

FIG. 2. Comparative structures of C termini of human myeloma proteins of all five classes. Note the extension of α and μ chains and the site of J chain attachment.

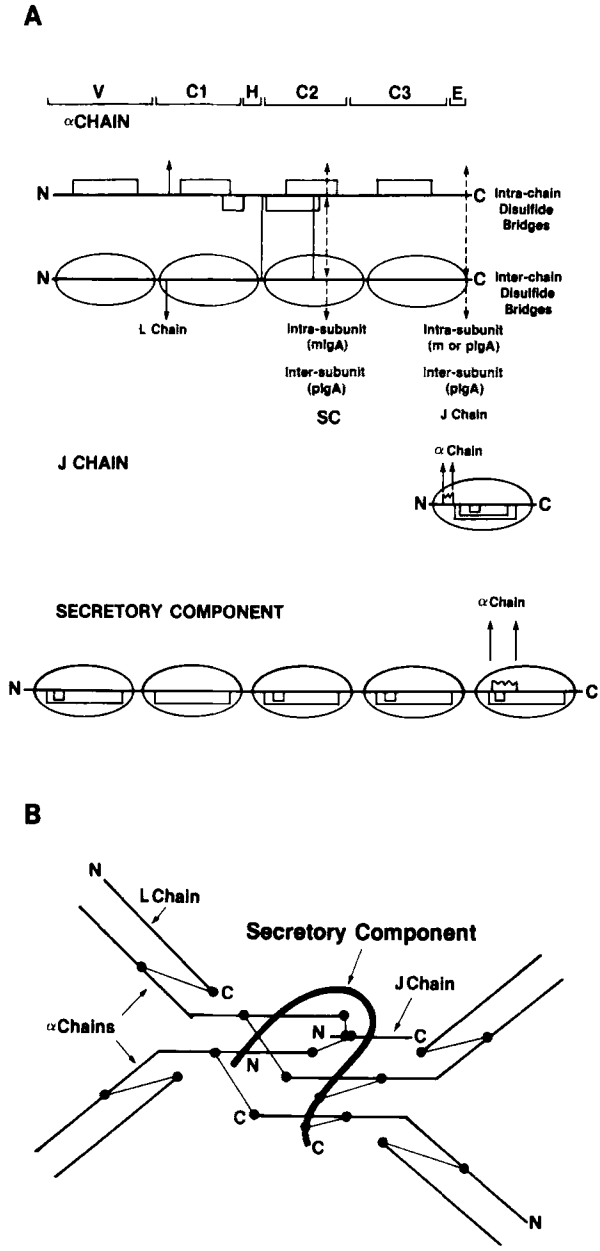

FIG. 3. Domain structures of component chains of the human S-IgA molecule with intra- and interchain disulfide bridges (A), and a tentative model of assembled S-IgA with interchain disulfide bridges (B). V, Variable and C1–3, constant region domains; H, hinge region; E, extension (see Fig. 2). (⌐¬), disulfide bridges; (⌐~¬), labile disulfide bridges; (⌐--¬), α- chain disulfide bridges involved in intra- or inter-α chain, J chain, and SC binding. For details and pertinent references see Section II,A–D.

1976; Lebreton *et al.*, 1976; Underdown and Schiff, 1986). Several polymeric IgA myeloma proteins have been described that appear to lack J chain completely or contain less than one J chain per polymer (Brandtzaeg, 1976b; Tomasi and Czerwinski, 1976).

The most remarkable structural differences between IgA_1 and IgA_2 molecules are located in the hinge region (Fig. 1). Selected peptide bonds in a unique sequence of 13 amino acids that are cleavable by bacterial IgA_1 proteases (Table I) allow the preparation of Fab and Fc fragments from IgA_1 proteins (Mehta *et al.*, 1973; Plaut *et al.*, 1974, 1975; Mulks and Plaut, 1978; Kornfeld and Plaut, 1981; Kilian *et al.*, 1979, 1980, 1983; Plaut, 1978, 1983; Mestecky and Kilian, 1985). There is a deletion in the corresponding segment of $\alpha 2$ chain, rendering proteins of IgA_2 subclass resistant to cleavage by commonly used bacterial IgA proteases. However, recent results indicate that Fab and Fc fragments from IgA_2 can be prepared with the use of unique proteases from *Clostridium* species that cleave IgA_1 as well as IgA_2 of the A2m(1) but not of the A2m(2) allotype (Fujiyama *et al.*, 1985). The structural basis for this unique sensitivity to proteases is apparent from Fig. 1. The hinge region of IgA_2 of A2m(1) but not of A2m(2) allotype contains a peptide bond, which is also present in IgA_1, and is located between prolyl (221) and valyl (222) residues; this bond is cleaved by clostridial proteases. Recently, a bacterial strain whose protease cleaved IgA_1 and IgA_2 of A2m(1) allotype was isolated from feces of a patient with ulcerative colitis and was identified as *Clostridium ramosum* (Senda *et al.*, 1985).

Bacterial proteases that cleave IgA_1 into Fab and Fc fragments are exquisitely specific: the only known substrate is human IgA_1 and IgA proteins from other species are resistant.

Further structural differences between IgA_1 and IgA_2 molecules have been observed in their carbohydrate compositions. There are statistically significant differences in the total carbohydrate content and the amounts of fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and mannose (Tomana *et al.*, 1976). The composition and localization of individual oligosaccharide side chains have been reported (Baenziger and Kornfeld, 1974a,b; Torano *et al.*, 1977) (Fig. 1). Further structural and functional differences between IgA_1 and IgA_2 subclasses have been recently reviewed (Mestecky and Russell, 1986).

B. J Chain

Extracellularly, this polypeptide is associated with polymeric IgA and IgM of many species but immunocytes producing other im-

TABLE I

BACTERIAL SPECIES PRODUCING IgA PROTEASES AND THEIR ASSOCIATION WITH INFECTIOUS DISEASES[a]

Disease	Species	Specificity, fragments produced
Meningitis, respiratory infections		
	Haemophilus influenzae	
	Neisseria meningitidis	IgA_1; Fab/Fc
	Streptococcus pneumoniae	
Urogenital infections		
	Neisseria gonorrhoeae	
	Ureaplasma urealyticum	IgA_1; Fab/Fc
Destructive periodontal disease		
	Bacteroides asaccharolyticus	IgA_1; Fc
	B. buccae	
	B. denticola	
	B. loescheii	
	B. melaninogenicus	
	B. oris	IgA_1; Fab/Fc
	Capnocytophaga gingivalis	
	C. ochracea	
	C. sputigena	
	Haemophilus parahaemolyticus	
Initial dental plaque formation, gingivitis(?)		
	Streptococcus mitior[b]	
	Streptococcus sanguis[b]	IgA_1; Fab/Fc; IgA without one Fab
Miscellaneous conjunctivitis (?)		
	Haemophilus aegyptius	IgA_1; Fab/Fc
	Gemella haemolysans	IgA_1; Fab/Fc
	Clostridium ramosum	IgA_1; Fab/Fc and IgA_2—A2m(1) allotype; Fab/Fc

[a] For details see Kilian *et al.* (1983), Mestecky and Kilian (1985), Fujiyama *et al.* (1985), and Senda *et al.* (1985).

[b] Only some strains produce IgA_1 protease.

munoglobulin isotypes may contain it (see Section IV,A). Comparative studies demonstrated remarkable structural similarities (for reviews see Inman and Mestecky, 1974; Koshland, 1975, 1985) with respect to the molecular weight, amino acid composition, and primary structure of human and mouse J chains (77% homology) (Mole *et al.*, 1977; Max and Korsmeyer, 1985; Cann *et al.*, 1982; Koshland, 1985). There are 137 residues with a high proportion of acidic amino acids, low numbers of glycine, phenylalanine, serine, and only 1 methionine. There are eight cysteine residues, six of which are involved in the formation of intrachain disulfides.

Two cysteines are connected to penultimate cysteine residues of α and μ chains (Mendez *et al.*, 1973; Mestecky *et al.*, 1974a; Mestecky and Schrohenloher, 1974). Although J chain and immunoglobulin chains display a low degree of sequence homology, recent data derived from computer-assisted analyses of sequences of human and mouse J chain and from circular dichroism measurements of denatured and renatured J chain suggested structural similarities to immunoglobulin domains (Zikan *et al.*, 1985). The profiles of the secondary structure of J chain were similar to those of superoxide dismutase or variable regions of immunoglobulin light chains, suggesting that the J chain folds into an eight-stranded anti-parallel β-sheet barrel. Experimental data obtained by circular dichroism measurements were in good agreement with computer analyses and indicated that J chain contains 37% β-sheet conformation with the rest of the structure as reverse turns (random coil) (Zikan *et al.*, 1985). Therefore, despite the differences between J chain and α or μ chain primary structures, the J chain apparently displays all β-sheet folding similar to that of an immunoglobulin domain. The possibility that J chain is arranged in an immunoglobulin-like fold further suggests that the interaction with the C4 or C3 domains during the process of polymerization and the SC-mediated transport of the J chain-containing polymeric IgA or IgM molecules through epithelial cells may be governed by interdomain associations (see below).

C. Secretory Component (SC)

Differences observed between the antigenic determinants of serum and secretory IgA led to the identification and isolation of an additional polypeptide chain in the S-IgA molecule (Hanson, 1961; Tomasi *et al.*, 1965) which is now designated as SC. It was demonstrated that in external secretions of normal individuals and of some IgA-deficient patients, IgM is present and associated with SC (Thompson, 1970; Brandtzaeg, 1975b; Arnold *et al.*, 1977, 1978). The essential role

of SC in the selective transport of IgA into external secretions was proposed and later confirmed in a variety of experimental systems (for review see Ahnen *et al.*, 1985; Brandtzaeg, 1981, 1985; Solari and Kraehenbuhl, 1985). Details of the interaction of polymeric IgA or IgM with SC and their transport will be described below.

SC is produced by epithelial cells found in secretory glandular tissues and on mucosal surfaces of gastrointestinal and respiratory tracts (Tourville *et al.*, 1969; Heremans, 1974; Brandtzaeg, 1985). Hepatocytes of certain mammalian species also produce SC as reviewed by Vaerman *et al.* (1982). Surprisingly, epithelial cells in Hassall's corpuscles of the thymus likewise contain SC (Tomasi and Yurchak, 1972) but further information concerning its biosynthesis and potential role is unavailable.

SC occurs in three molecular forms: (1) as a membrane protein on surfaces of epithelial cells and hepatocytes where it functions as a receptor for polymeric immunoglobulins, (2) as a component chain of S-IgA and S-IgM, and (3) as a free glycoprotein found in several external secretions (Heremans, 1974; Brandtzaeg, 1985).

Subsequent studies demonstrated that free SC (1) interacts *in vitro* specifically with polymeric IgA and IgM because of the presence of an SC-binding site on molecules (see below), (2) stabilizes their quaternary structure (Jerry *et al.*, 1972; Heremans, 1974; Mestecky *et al.*, 1974b; Brandtzaeg, 1977, 1985; Murkofsky and Lamm, 1979), and may increase their resistance to various proteolytic enzymes (Lindh, 1975).

Depending on the technique used for the determination, the molecular weight of SC ranged from approximately 50,000–90,000 (Tomasi and Bienenstock, 1968; Kobayashi, 1971; Heremans, 1974; Brandtzaeg, 1985). Recent structural studies of rabbit (Mostov *et al.*, 1984) and human (Eiffert *et al.*, 1984) SC indicate that it consists of a single polypeptide chain with 549 to 558 amino acids and large amounts of carbohydrate (20%) (Heremans, 1974; Tomana *et al.*, 1978) attached by N-glycosidic bonds to asparagine residues as 5–7 oligosaccharide side chains (Purkayastha *et al.*, 1979; Mizoguchi *et al.*, 1982).

Human SC displays a characteristic amino acid composition (Kobayashi, 1971) with the 20 cysteine residues involved in the formation of 10 intrachain disulfide bridges (Eiffert *et al.*, 1984). The total absence of methionine renders the molecule resistant to the chemical cleavage by cyanogen bromide (Kobayashi, 1971; Lamm and Greenberg, 1972; Mestecky *et al.*, 1974c). However, SC, particularly in its unbound form, is highly susceptible to cleavage by proteolytic enzymes (Brandtzaeg, 1975c).

The determination of the complete covalent structure of rabbit (Mostov *et al.*, 1984) and human (Eiffert *et al.*, 1984) SC resulted in the elucidation of its secondary structure and the probable structural basis of its selective reactivity with polymeric J chain-containing immunoglobulins.

These studies of the primary structure of rabbit SC by DNA sequence analyses and human free SC by classical protein sequencing demonstrated remarkable similarities in their general structures. When compared, the rabbit SC and the human analog display homology of about 60%. Based on the primary structures of rabbit and human SC, it became obvious that the entire polypeptide chain may be subdivided into 5 sequential homology regions, each comprising 104–114 amino acid residues. The five homology regions of human SC are shown in Fig. 3.

The homology of the regions is quite remarkable so that many amino acid residues recur in all regions in the same position. Particularly striking is the periodic distribution of the 20 cysteine residues and their involvement in disulfide bridges. Each homology region, with the exception of the second, contains 2 intrachain disulfides: a "large bridge" connecting 2 cysteine residues that are approximately 70 amino acids apart is present in all homology regions; and a "small bridge" connecting 2 cysteine residues separated by 7 amino acid residues, found in all homology regions except the second. The fifth homology region contains an additional intrachain disulfide bond (between residues 467 and 501) that is easily cleaved by partial reduction (Cunningham-Rundles and Lamm, 1975; Eiffert *et al.*, 1984). This labile disulfide bond is involved in the formation of a disulfide bridge that links SC to the α chains of one of the monomers in dimeric IgA, apparently through cysteine residue 311 on the α chain (see below). There are no free sulfhydryl groups on SC. The fifth homology region is shorter and displays considerable variability with respect to the C-terminal amino acid residue due to the apparently irregular proteolytic cleavage which occurs when the membrane-bound form of SC is released (see below). The fifth region displays less homology with the first four regions of SC.

As described above, human free SC and rabbit polyimmunoglobulin receptor (corresponding to the membrane form of SC) show a 60% homology. Cysteine residues are similarly distributed and one may therefore anticipate an analogous distribution of disulfide bridges in rabbit SC. Mostov *et al.* (1984) demonstrated that homology regions of rabbit SC are structurally related to immunoglobulins, particularly to their variable domains. Homology regions of human SC also show this

clear homology with the variable domain of the immunoglobulin L chain (Eiffert *et al.*, 1984). Immunoglobulin domains and SC homology regions exhibit similar length and contain cysteine residues in an intrachain disulfide bridge with a 70 amino acid residue loop. Computer analyses of the primary structure led to the prediction that the secondary structure of human SC will be analogous to that of immunoglobulin domains. Based on these results, the authors proposed that the tertiary structures of both proteins will be analogous.

It should be remembered that the secondary structure of J chain also exhibits immunoglobulin-like folding (Zikan *et al.*, 1985) and that the interactions of the constant-region domains of α, μ, and J chains with SC may be governed by the principles of their domain-like structures and complementarities.

D. Interaction of Component Polypeptide Chains in the Secretory IgA Molecule

Structural and stoichiometric analyses of rabbit and human S-IgA revealed that the typical 11 S molecule contains two monomeric subunits, one J chain and one SC (molecular formula $(\alpha_2L_2)_2$.J.SC (O'Daly and Cebra, 1971; Mestecky *et al.*, 1972a). The number of J chains present in the secretory IgA molecule has been controversial. Using various techniques, most investigators found one J chain per dimeric S-IgA (O'Daly and Cebra, 1971; Halpern and Koshland, 1973; Mestecky *et al.*, 1972b; Zikan *et al.*, 1986). However, based on the number of J chains found in myeloma IgA proteins and polyclonal IgA dimers, Brandtzaeg (1975a, 1976a) suggested that there are two J chains in S-IgA. Human polymeric paraproteins of IgA or IgM classes apparently vary greatly with respect to their J chain content: some may lack this polypeptide (Brandtzaeg, 1976b; Eskeland and Brandtzaeg, 1974; Tomasi and Czerwinski, 1976), other paraproteins may have more than one J chain (Mihaesco *et al.*, 1973; Ricardo and Inman, 1974; Brandtzaeg, 1975a; Grubb, 1978). The structural basis of this apparent variability remains unexplained. In addition to the dimeric 11 S S-IgA, human external secretions contain tetrameric 15.5 S S-IgA molecules (Zikan *et al.*, 1972; Halpern and Koshland, 1973). Stoichiometric analyses of tetrameric S-IgA isolated from human colostrum also indicated the presence of a single J chain (Halpern and Koshland, 1973).

Penultimate cysteine residues of α and μ chains form disulfide bridges with J chain (Mendez *et al.*, 1973; Mestecky *et al.*, 1974a; Mestecky and Schrohenloher, 1974) which interacts through apparently weak noncovalent bonds with the Cμ4 or Cα3 domains. Unlike H and L chains, J chain is released from mildly reduced or S-sulfona-

ted polymers even in the absence of dissociating solvents (Mestecky *et al.*, 1972a; Brandtzaeg, 1975d). Whether J chain connects two monomeric subunits (Wilde and Koshland, 1973; Garcia-Pardo *et al.*, 1981) or is linked to penultimate cysteine residues within one monomer (Mestecky, 1976; Lebreton *et al.*, 1976; Underdown and Schiff, 1986) remains controversial and further studies will be required for definitive determination. The fact that the antigenic determinants of J chain are not easily accessible in polymeric IgA, IgM (Inman and Mestecky, 1974), and particularly in S-IgA (Kutteh *et al.*, 1982a) suggests that J chain is well hidden in these molecules.

Stoichiometry of 11S S-IgA led to the finding of 1 SC per dimer (Mestecky *et al.*, 1972a). This conclusion was based on the homogeneity of the distribution of antigenic determinants of SC in human colostral S-IgA (Mestecky *et al.*, 1970) and the proportion of SC to α chains (one to four) in reduced or S-sulfonated S-IgA examined by electrophoresis in the presence of sodium dodecyl sulfate and urea (Mestecky *et al.*, 1972a).

Both additional chains, SC and J, are covalently bound in human S-IgA to the Fc region (Zikan *et al.*, 1972; Mestecky *et al.*, 1974a). Although the cysteine residues involved in the formation of disulfide bridges between α and J chain have been identified (Mendez *et al.*, 1973; Mestecky *et al.*, 1974a) the site of SC attachment remains uncertain (see below).

In vitro experiments convincingly demonstrated that SC binds only to polymeric IgA or IgM (Mach, 1970; Radl *et al.*, 1971; Brandtzaeg, 1974a, 1976b; Weicker and Underdown, 1975; Lindh and Bjork, 1976a,b; Kühn and Kraehenbuhl, 1979). The discovery of J chain in these immunoglobulins (Halpern and Koshland, 1970; Mestecky *et al.*, 1971) led Mach (1970, addendum to the reprint) to postulate that this small polypeptide is instrumental in SC binding. Despite the dissenting opinion (Tomasi and Czerwinski, 1976) based on the ability of J chain-deficient human polymeric IgA to bind bovine SC, Brandtzaeg (1985) has maintained that SC binding by polymeric IgA and IgM is dependent on the presence of J chain: pentameric IgM that lacked J chain did not bind SC efficiently (Eskeland and Brandtzaeg, 1974) and the ability of polymeric IgA to bind SC in solution or on the surface of SC-bearing epithelial cells was related to the J chain content (Brandtzaeg, 1976b; Brandtzaeg and Prydz, 1984). Polyclonal or monoclonal polymeric IgA that contained 4.9 mg or more J chain per 100 mg IgA bound SC in quantities almost 10-fold greater than monoclonal monomers or dimers that contained 0.4 mg or less of J chain/100 mg IgA. It is not clear, however, how the presence of J chain leads to

such remarkable enhancement of SC binding. J chain obtained from polymeric IgA by mild reduction did not substantially influence the complexing of SC to polymeric IgA (Brandtzaeg, 1975e), suggesting that the interaction between SC and J chain is rather weak. However, free J chain is known to dimerize spontaneously (Inman and Mestecky, 1974) and therefore may not react with SC as strongly as a single J chain present in a dimeric IgA molecule because of the J chain self-association which would preclude binding to SC. The role of J chain in the formation of an SC-binding site on polymeric IgA has been recently questioned by Schiff *et al.* (1986a): SC-binding and SC-nonbinding dimeric IgA contained approximately the same amounts of J chain as estimated by the radioactivity found in the region of electrophoretic migration of J chain on alkaline urea gel (Reisfeld and Small, 1966). The authors suggested that the conformational requirement for IgA to express SC-binding activity may be missed during polymer assembly even when J chain is present. Furthermore, a tyrosine residue, whose localization has not been determined, appeared to play a critical role in maintaining the SC binding site on polymeric IgA. Validation of these conclusions will require further experiments. The content of J chain in SC-binding and nonbinding polymeric IgA needs also to be determined by assays that are based on the antigenic determinants of this polypeptide. Detection of a protein with a characteristically fast electrophoretic mobility on alkaline urea gels does not necessarily indicate the presence of J chain: α1-antitrypsin or serum albumin, both of which form complexes with polymeric IgA, may mimic J chain because of their identical mobilities on alkaline urea gel electrophoresis (Tomasi and Hauptman, 1974). Furthermore, the frequently observed ability of polymeric IgA to complex with a wide range of serum proteins may substantially reduce its SC-binding capacity. Nevertheless, an irregularity in the IgA polymer assembly, even in the presence of J chain, is a distinct possibility. Although the conformational changes induced chemically by radioiodination of tyrosine residues in the antigen-binding site of antibodies interfere with their ability to bind a corresponding hapten (Klostergaard *et al.*, 1978), it is surprising that this procedure would have such a profound effect on the SC-binding activity of polymeric IgA.

Previous structural studies of human S-IgA cleaved by cyanogen bromide indicated that J chain and SC are bound to different fragments of the α chain and are not connected by disulfide bridges (Mestecky *et al.*, 1974c). Furthermore, reduction (Kobayashi, 1971) or S-sulfitolysis (Mestecky *et al.*, 1972a) of human S-IgA resulted in the release of substantial amounts of J chain and of SC under nondisso-

ciating conditions. Therefore, the presence of intact disulfide bridges appears to be essential in the formation of an SC-binding site in the dimeric IgA molecule. The *in vitro* affinity of both polymeric IgA and IgM for SC has been studied by many investigators and, generally, pentameric IgM displayed stronger noncovalent interactions with SC than dimeric IgA as summarized by Brandtzaeg (1985). Although different polymeric IgA or IgM proteins are quite heterogeneous with respect to their SC-binding activity, the structural basis of the higher affinity of IgM than IgA for SC is not clear. Brandtzaeg (1985) speculated that pentameric IgM binds SC with higher affinity due to its higher content of J chain (at least three per pentamer); this latter point is not universally accepted because other investigators, using several quantitative methods, found only one J chain per pentameric IgM (Mestecky *et al.*, 1971, 1972b; Mihaesco *et al.*, 1973; Koshland and Wilde, 1974; McCumber and Clem, 1976). The affinity of polymeric immunoglobulins for SC is of comparable magnitude to that observed for antigen–antibody reactions. A K_a of 10^8 M^{-1} or higher has been obtained in different experimental systems (Brandtzaeg, 1985).

The ability of SC to bind polymeric immunoglobulins is also demonstrable in plasma cells of various organs (Brandtzaeg, 1973) and is remarkably preserved, despite the use of fixatives (ethanol) and procedures (paraffin embedding at temperatures often higher than 50°C and repeated exposure to xylene and ethanol) that must lead to substantial denaturation. As described below, an SC-binding test performed on sections of various tissues and suspensions of fixed lymphoid cells has been used for the detection of cells that produce polymeric IgA or IgM (Brandtzaeg, 1973; Radl *et al.*, 1974; Crago and Mestecky, 1979).

Recent results concerning the structure of IgA, SC, and J chain indicate that both SC and J chain display immunoglobulin domain-like folding of their polypeptide chains and belong to the immunoglobulin superfamily (Mostov *et al.*, 1984; Eiffert *et al.*, 1984; Zikan *et al.*, 1985). Therefore, it is highly probable that the mutual interactions between the Fc portion of dimeric IgA, J chain, and SC are based on the complementarities of their domain-like structures. Beale (1985) proposed a model for the interaction of polymeric IgA or IgM with SC that is based on the differences in proteolytic fragmentation of free vs IgA-bound SC. In this model the domain-like structures are arranged in a circular pattern with a partial overlap of the first and fifth domains to allow for an interdomain disulfide bridge. Because certain interdomain peptide bonds in SC become resistant to proteolysis, the author speculates that they are inaccessible due to their interaction with the Fc part of IgA or IgM. Furthermore, it was predicted that at least three

of the five SC domains interact with polymeric IgA or IgM and that in the case of IgM the SC binds at the planar surface of the central $(Fc)_{5\mu}$ disk.

This model must be reconsidered in the light of recent results concerning the primary and predicted secondary structures of all component polypeptides involved in the formation of the SC-binding site on polymeric, J chain-containing IgA. Because SC is composed of five immunoglobulin-like domains it is likely that these will interact with four Cα3 domains of dimeric IgA and J chain. After the initial noncovalent interaction, this complex becomes stabilized through the formation of disulfide bridges (Brandtzaeg, 1977). It has been previously shown that SC forms disulfide bridges with only one of the monomeric subunits of dimeric IgA (Underdown, 1975; Underdown *et al.*, 1977; Garcia-Pardo *et al.*, 1979) and not with J chain (Mestecky *et al.*, 1974c). Recent results derived from primary structures of human (Eiffert *et al.*, 1984) and rabbit (Mostov *et al.*, 1984) SC strongly suggest that a labile disulfide bridge which could interact with cysteine residues on α chains is localized in the fifth domain of SC (between cysteine residues 467–501 of human and 464–497 of rabbit SC). The cysteine residues forming this labile disulfide bond are likely to react with cysteine residues 311 in Cα2 domains as proposed by Hanly *et al.* (1985). The same authors based their model of SC-IgA interaction on the assumption that cysteine residues 311 in the Cα2 domains form a labile disulfide bridge due to a novel type of Cα2 and Cα3 domain interactions in the Fc part of the polymeric IgA molecule. In contrast to corresponding Cγ2 domains of the γ chains, which have no close contact (Munn, *et al.*, 1971), Cα2 domains display a crossing pattern that would bring cysteine residues 311 from two α chains in close proximity. The arrangement of Cα3 domains, which appear to be the main site of noncovalent interactions with SC, may be different in monomeric IgA. Although in monomeric IgA Cα3 domains may be in close contact, the incorporation of J chain into IgA during the intracellular polymerization could result in a structurally alternative arrangement of Cα3 domains which, together with J chain, would form an SC-binding site. Another plausible explanation for the formation of an SC-binding site on polymeric IgA may be based on the increased affinity of four Cα3 domains and J chain vs only two Cα3 domains in monomeric IgA. However, this explanation is improbable because monoclonal monomeric or J chain-deficient polymeric IgA bound SC to the same degree (Brandtzaeg, 1976b). Therefore, it appears that the structural modification in the arrangement of Cα3 domains which is elicited by the incorporation of J chain into the polymeric structure

creates a site to which SC binds on the basis of complementarities of their domain structures. The role of carbohydrate side chains of SC, J chain, and Fcα in these interactions has not been studied. It is, however, interesting that SC contains a remarkably large amount of carbohydrates, linked by N-glycosidic bonds as five to seven oligosaccharide side chains (see above). J chain contains 8% carbohydrates (Niedermeier *et al.*, 1972), as one oligosaccharide side chain (Baenziger, 1979). Although Cα3 domains do not possess a carbohydrate moiety, there is an oligosaccharide side chain linked to asparagine residue 459 (Torano *et al.*, 1977). This residue is present in a segment by which α or μ chains extend over the C termini of γ and ε chains and which also contain cysteine residues in both α and μ chain involved in the formation of a disulfide bond with J chain. An analogous oligosaccharide chain is present in the same segment of the μ chain (Torano *et al.*, 1977). Further studies will be required to determine whether the carbohydrate moieties present in abundance on all component chains play any role in the interaction of α or μ chains with J chain and subsequently with SC.

III. Interaction of IgA with Nonlymphoid Cells

IgA of various molecular forms and IgA-containing immune complexes interact with morphologically and functionally diverse cells. As described in Table II, these include T, B, and NK cells, polymorphonuclear leukocytes (PMN), macrophages (Mϕ) from various body fluids and tissues, epithelial cells that cover mucous membranes or are present in external secretory glands, hepatocytes, and erythrocytes (Mestecky *et al.*, 1987). The interactions with such cells profoundly influences the biosynthesis of IgA, its distribution in various body fluids, and the effector functions of IgA.

A. Phagocytic Cells

The presence of receptors for the Fc part of heterologous as well as homologous IgA (RFcα) on a subpopulation of human peripheral blood PMN and Mϕ was detected by Spiegelberg *et al.* (1974) and Fanger *et al.* (1980, 1981, 1983a,b). Although the RFcα has not been biochemically characterized, it is distinct from RFcγ. Further studies revealed that RFcα interacts with the Cα2 domains of polymeric and monomeric IgA of both subclasses and with S-IgA (Fanger *et al.*, 1981). The expression of RFcα is markedly enhanced by incubation of PMN with both myeloma and S-IgA. In contrast to incubation with IgG, which did not affect RFcα expression, incubation with either IgA

TABLE II

INTERACTIONS OF IgA WITH LYMPHOID AND NONLYMPHOID CELLS[a]

Cell type and source	IgA form	Specificity	Receptor	Function	References
Polymorphonuclear leukocytes (PMN)	mIgA	Fc	?		Spiegelberg *et al.* (1974); Fanger *et al.* (1980, 1981)
Human	pIgA	*Cα2 domain*	*Pronase sensitive*		
	S-IgA				
	IgA_1				
	IgA_2				
Peripheral Blood			Different from Fcγ		
Oral					
				Removal of IgA-containing mixed immune complexes	Tomino *et al.* (1982) Moldoveanu *et al.* (1985a)
				Antibody-dependent cell-mediated cytotoxicity	Shen and Fanger (1981); Fanger *et al.* (1983a,b)
				IgA catabolism (?)	Moldoveanu *et al.* (1985a)
				Phagocytosis enhancement	Fanger *et al.* (1983)
Milk				IgA in milk PMN	Crago *et al.* (1979); Laven *et al.* (1981); Moro *et al.* (1983)
				Release of S-IgA from milk PMN in the intestine of a newborn	Weaver *et al.* (1981, 1982, 1984)

(*continued*)

TABLE II (*continued*)

Cell type and source	IgA form	Specificity	Receptor	Function	References
Macrophages (Mϕ)	mIgA	Fc	?		Fanger *et al.* (1980, 1981)
Human	pIgA				
	S-IgA				
Mϕ cell line		Fc			Maliszewski *et al.* (1985)
Milk				IgA in milk cells	Crago *et al.* (1979); Laven *et al.* (1981); Moro *et al.* (1983)
				Release of S-IgA from milk Mϕ; vehicles of S-IgA transport	Pittard *et al.* (1977); Weaver *et al.* (1982, 1984)
				IgA-dependent monocyte-mediated cytotoxicity and antibacterial activity	Lowell *et al.* (1980); Shen and Fanger (1981)
Mouse (spleen)	S-IgA	Fc	?		Tagliabue *et al.* (1984)
Mouse (Kupffer cells)	pIgA- or mIgA-containing immune complexes	For immune complexes	?	Removal of circulating immune complexes	Rifai and Mannik (1984)
Erythrocytes	mIgA	Fc	?	?	Mota (1982)
Human	pIgA				
	S-IgA				
	IgA_1				
	IgA_2				
	Mixed immune complexes (IgA,IgG,C3)	C3	Cr1	Removal of circulating mixed immune complexes	Waxman *et al.* (1986); Matsuda *et al.* (1986)

Epithelial cells Human, rabbit, mouse, rat, and other species	pIgA	Fc	Secretory component	Selective transport of pIgA into external secretions	Heremans (1974)[b]; Brandtzaeg (1985); Ahnen *et al.* (1985)
Hepatocytes Rat, mouse, rabbit	pIgA	Fc	Secretory component	Selective hepatobiliary transport of pIgA and pIgA-containing immune complexes. Enforcement of intestinal immunity and removal of immune complexes	Vaerman *et al.* (1982)[b]; Brown *et al.* (1983); Underdown and Schiff (1986); Sancho *et al.* (1986)
Rat, mouse, human	mIgA pIgA S-IgA IgA_1 IgA_2	Galactose residues	Asialoglycoprotein receptor	Catabolism of IgA; hepatobiliary transport of IgA	Stockert *et al.* (1982); Phillips *et al.* (1984); Tomana *et al.* (1985); Schiff *et al.* (1986); Underdown and Schiff (1986)
NK cells Human	S-IgA $pIgA_2$	Fc	?	Inhibition of NK cell activity	Komiyama *et al.* (1986)
Lymphocytes[a]					
Human ($T4^+$)	Serum IgA	Fc	?	Antibacterial activity	Tagliabue *et al.* (1985)
Mouse (Thy 1.2^-)	S-IgA Monoclonal serum IgA	Fc			Tagliabue *et al.* (1984)

[a] Including IgA-mediated effector functions but excluding immunoregulatory functions mediated by $T_{Fc\alpha}$ and $B_{Fc\alpha}$ cells (see Section IV,E).

[b] Only review articles are listed; for details see Section III,C and D.

preparation resulted in increased numbers of RFcα per cell as well as an increase in the total number of PMN expressing RFcα (Fanger *et al.*, 1983a,b). This finding may be relevant to the increased numbers of RFcα detected on PMN obtained from mucosal surfaces. The authors speculate that PMN migrating from the blood to mucosal surfaces are exposed to greater levels of locally produced IgA and consequently express more RFcα (Fanger *et al.*, 1983a,b). This, in turn, may explain why PMN from mucosal surfaces display more phagocytic activity than PMN from peripheral blood. Although free or erythrocyte-complexed IgA bound to surfaces of PMN from peripheral blood were not significantly internalized and did not trigger phagocytosis, in concert with suboptimal quantities of anti-erythocyte IgG enhanced phagocytosis was observed (Fanger *et al.*, 1983a,b). Interestingly, PMN from the oral cavity could phagocytize through IgA alone. The ability of human peripheral blood PMN to bind and internalize various preparations of myeloma IgA and IgA-containing immune complexes was also studied with the use of immunochemical and immunohistochemical techniques. Polymeric forms of IgA_1 and IgA_2 colloidal gold-labeled paraproteins were bound and internalized but monomers were not (Moldoveanu *et al.*, 1985a). However, analyses of internalized IgA indicated that most of the IgA was present in monomeric form, suggesting the dissociation of polymers.

Peripheral blood PMN from patients with diseases with increased levels of serum IgA and IgA-containing immune complexes (such as IgA nephropathy and liver cirrhosis) contain intracellular IgA together with IgG and C3 as disclosed by immunofluorescence (Tomino *et al.*, 1982). These findings indicate that immune complexes composed of IgA, IgG, and C3 were internalized. Quantitation of IgA in PMN showed higher levels of this immunoglobulin in six cirrhotic patients than in normal controls. Interestingly, PMN from 2 IgA_1 myeloma patients also contained more IgA than PMN from normal controls (Moldoveanu *et al.*, 1985a). This finding suggests that PMN exposed to high levels of IgA can bind as well as internalize this immunoglobulin in free form without its involvement in immune complexes. This is apparently the reason for the presence of high levels of S-IgA in PMN and Mϕ isolated from human colostrum and milk (Laven *et al.*, 1981). A high percentage of cells in these fluids stained intensely with fluorochrome-labeled anti-IgA reagents, and immunoelectron microscopy revealed the presence of vacuoles rich in IgA-containing material (Crago *et al.*, 1979; Moro *et al.*, 1983). IgA from lysates of colostral cells occurred mostly in polymeric form with SC attached (Laven *et al.*, 1981).

The presence of receptors for IgA or IgA-containing immune complexes on Mϕ has been demonstrated in various experimental systems. In contrast to an earlier study of Huber *et al.* (1971), in which erythrocytes coated with various IgA preparations failed to bind human peripheral blood monocytes and promote phagocytosis, Fanger *et al.* (1980) demonstrated the presence of receptors for Fcα on these cells by using an analogous experimental system. In a subsequent communication (Fanger *et al.*, 1981) it was shown that IgA receptors bind polymeric as well as monomeric IgA of both subclasses and that the binding site is localized in the Cα2 domain.

In a recent study, Sancho *et al.* (1986) demonstrated the presence of receptors for Fc of both monomeric and polymeric IgA on murine Kupffer cells. A unique receptor specific for IgA-containing immune complexes but not for free IgA has also been detected on murine Kupffer cells (Rifai and Mannik, 1984). This receptor recognizes large, covalently stabilized immune complexes composed of several molecules of monomeric and/or polymeric IgA. The biological importance of Fcα receptors on phagocytic cells is not entirely understood. Although IgA is generally considered as a poor opsonin (Heremans, 1974; Wilton, 1978), PMN from the oral cavity effectively phagocytized particles coated only with IgA (Fanger *et al.*, 1983a,b).

The presence of large quantities of S-IgA within colostral and milk phagocytes (Pittard *et al.*, 1977; Crago *et al.*, 1979) also indicates that this isotype can be internalized by PMN and Mϕ found in secretions of glands that locally produce large amounts of S-IgA. It has been proposed that colostral and milk Mϕ serve as vehicles for the delivery of S-IgA into milk (Pittard *et al.*, 1977) and to the lower gastrointestinal tract of breast-fed infants. S-IgA released from these cells could therefore reinforce the protection of the newborn intestine (Weaver *et al.*, 1982, 1984).

Detection of IgA, IgG, and C3 in peripheral blood PMN suggests that these cells may be involved in the removal and catabolism of immune complexes from the circulation. Furthermore, a novel receptor detected on murine Kupffer cells and specific for IgA immune complexes appears to be involved in their effective removal from the circulation (Rifai and Mannik, 1984). The possibility that PMN and Mϕ may be also involved in the catabolism of free circulating IgA deserves further attention.

IgA purified from sera of patients with disseminated meningococcal disease induced monocyte-mediated anti-meningococcal activity in the absence of complement (Lowel *et al.*, 1980; Griffiss, 1983). The authors suggested that IgA-dependent, monocyte-mediated, antibac-

terial activity is physiologically relevant to mucosal sites which are rich in IgA but functionally deficient in complement components.

S-IgA or serum IgA by itself or in synergy with IgG has been shown to mediate IgA-dependent antibacterial activity of PMN, Mϕ, and mouse (Thy 1.2^-) or human ($T4^+$) lymphocytes from peripheral blood, spleen, and Peyer's patches (Shen and Fanger, 1981; Tagliabue *et al.*, 1984, 1985). These data indicate that IgA can direct and "arm" various populations of cells effective in cell-mediated antibacterial activity. In contrast, S-IgA or a polymeric myeloma IgA_2 paraprotein in physiologic concentrations inhibited the NK cell activity of peripheral blood mononuclear cells against chromium-labeled K562 target cells (Komiyama *et al.*, 1986).

B. Erythrocytes

Receptors for Fc of human IgA of various molecular properties, including S-IgA, monomeric and polymeric IgA, and IgA_1 and IgA_2, have been detected on surfaces of human, rabbit, and sheep erythrocytes by agglutination and complement-mediated hemolysis (Mota, 1982). For optimal binding, erythrocytes were incubated with a highly concentrated solution of IgA (4 mg/ml). Only a minute proportion of IgA interacted with erythrocytes; on an average 4×10^4 IgA molecules were bound per cell.

IgA was also detected on erythrocytes from peripheral blood of some IgA nephropathy patients, whose serum contained mixed IgA, IgG, and C3 immune complexes (Matsuda *et al.*, 1986). Such immune complexes partially purified by polyethylene glycol precipitation bound to the surface of erythrocytes obtained from an IgA-deficient patient, although isolated myeloma IgA of various molecular properties did not. Recently, Waxman *et al.* (1986) demonstrated that primate erythrocytes display a receptor for the C3b component of complement (CR1) which binds immune complexes containing mouse IgG_1 antibodies and C3; mouse IgA-containing immune complexes were bound relatively poorly. When infused into arterial circulation, IgA immune complexes were taken up by kidney and lung. In contrast, erythrocyte-bound IgG_1-containing immune complexes were primarily cleared in the liver and spleen. Therefore, it appears that IgA found on erythrocytes of IgA nephropathy patients occurs in a form of mixed immune complexes that are bound through CR1 receptors and that isolated IgA is not efficiently bound by human erythrocytes.

C. Epithelial Cells

It has been amply documented that epithelial cells lining surfaces of gastrointestinal and respiratory tracts, acinar and ductal cells of

various external secretory glands, and uterine lining are instrumental in the selective transport of polymeric IgA into external secretions (Tomasi *et al.*, 1965; Heremans, 1974; Ahnen *et al.*, 1985; Brandtzaeg, 1985; Solari and Kraehenbuhl, 1985). Immunochemical studies of S-IgA molecules and immunohistochemical examinations of secretory tissues indicated that the product of epithelial cells—SC—associated in S-IgA with polymeric IgA, is intimately involved in the selective transport. Because various models of IgA transport have been extensively reviewed in recent literature (Brandtzaeg, 1981, 1985) limited information is provided here. Based on immunohistochemical examinations of fixed sections of secretory tissues, Brandtzaeg (1974b) proposed a model of IgA transport in which SC functioned as a surface receptor for polymeric J chain-containing IgA or IgM on various epithelial cells. The validity of this concept was established in tissue sections by Brown *et al.* (1976, 1977) and *in vitro* by Crago *et al.* (1978) and Nagura *et al.* (1979), who used a colonic carcinoma cell line (HT-29) which expresses SC on its surface (Huang *et al.*, 1976), and by Brandtzaeg (1978) who used normal epithelial cells dissociated from intestinal mucosa.

Evidence for the identity of SC as a receptor for polymeric IgA was based on the findings that (1) proteolytic digestion of living cells led to the loss of IgA binding and reexpression of SC-restored binding activity, (2) anti-SC blocked IgA binding, and (3) S-IgA which contains SC was not bound (Crago *et al.*, 1978; Nagura *et al.*, 1979). Brandtzaeg (1978) observed in immunofluorescence tests with two fluorochromes that IgA and SC displayed concurrent membrane distribution on isolated epithelial cells from normal colon mucosa. In a subsequent study with the subcloned HT-29 colonic carcinoma cell line, Brandtzaeg and Prydz (1984) further refined this transport model and demonstrated that SC is the receptor for polymeric IgA or IgM and that the binding is related to the J chain content in these polymeric immunoglobulins. Biochemical, biosynthetic, and functional aspects of membrane SC have been extensively studied in several laboratories (Mostov *et al.*, 1980, 1984; Kühn and Kraehenbuhl, 1981; Mostov and Blobel, 1982; Kühn *et al.*, 1983; Solari and Kraehenbuhl, 1984). mRNA for SC isolated from rabbit mammary gland and translated in a cell-free system yielded a product which was considerably larger than free SC found in milk or bile (Mostov *et al.*, 1980). Subsequent experiments revealed that this larger product represented the transmembrane form of SC (Kühn and Kraehenbuhl, 1981) which is synthesized on the rough endoplasmic reticulum. After glycosylation is completed in the Golgi complex, SC is directed to the basolateral membrane and inserted as a membrane form of SC where it acts as a receptor for

polymeric IgA or IgM. Sequence studies of DNA complementary to SC mRNA revealed that rabbit SC consists of 773 amino acids with an 18 amino acid N-terminal signal peptide, a 629 amino acid extracellular SC, a 23 amino acid membrane spanning segment, and a 103 amino acid cytoplasmic tail (Mostov *et al.*, 1984). Structural characteristics of extracellular SC have been described above (SC structure). Analogous studies using the human HT-29 .E10 cloned colonic carcinoma cell line also showed that the transmembrane form of SC which acts as a receptor for polymeric immunoglobulins is larger than free SC (100 kd vs 80 kd, respectively). Time-course biosynthetic experiments showed that free SC is found in culture supernatants 1–2 hours after the synthesis of the membrane form on rough endoplasmic reticulum (Solari and Kraehenbuhl, 1984).

The membrane form of SC binds polymeric IgA or IgM apparently by noncovalent interactions; the exact point at which the SC–polymeric IgA complex is stabilized by disulfide bridges is at present unknown. Studies suggest that a free sulfhydryl group on polymeric IgA initiates a disulfide bond exchange reaction by reducing a reactive disulfide bond on SC (Cunningham-Rundles and Lamm, 1975; Lindh and Bjork, 1976a; Murkofsky and Lamm, 1979; Eiffert *et al.*, 1984).

Further experiments suggested that a disulfide bond exchange enzyme(s), isolated from rat liver, promotes the covalent interaction between human SC and polymeric IgA (Murkofsky and Lamm, 1979). Whether an analogous enzyme is found in human epithelial cells has not been determined. However, it is likely that formation of covalent bonds which may be initiated on the cell membrane proceeds during the intracellular transport of the SC–polymeric IgA complex with the participation of disulfide bond exchange enzymes. The possible role of carbohydrates, found in high amounts on both SC and IgA, in SC–IgA interactions has not been determined; preliminary results indicate that a partial removal of carbohydrates has no effect (Tomana, unpublished observations).

The membrane SC–polymeric IgA complex is internalized and transported in the form of a vesicle, observed earlier by immunofluorescence and electron microscopy (O'Daly *et al.*, 1971; Brown *et al.*, 1976; Nagura *et al.*, 1979), through the epithelial cell. This transcytoplasmic transport involves microtubules and can be inhibited by colchicine (Nagura *et al.*, 1979). Intracytoplasmic, IgA-containing vesicles are moved toward the apical surface of epithelial cells and fuse with the apical membrane. The SC–IgA complex is released in the form of a completed S-IgA molecule. Because S-IgA-bound SC or free

SC is considerably smaller than its transmembrane form, proteolytic cleavage of SC that results in the release of the S-IgA molecule must occur. The exact site of SC cleavage (within intracytoplasmic vesicles or at the time of their fusion with the apical membrane) and proteolytic enzyme(s) involved have not been determined. Based on the heterogeneity of the amino acids at the C terminus of free SC (Eiffert *et al.*, 1984) isolated from human colostrum, it is probable that several proteolytic enzymes may be involved. Using the HT-29 colonic carcinoma cell line, Nagura *et al.* (1979) determined that the transcellular transport of IgA took approximately 30 minutes.

The SC-mediated transepithelial transport of polymeric IgA represents a unique system of interaction between a receptor and its ligand (Fig. 4). SC is produced by epithelial cells irrespective of the presence of its ligand, (polymeric IgA or IgM) and it is found in many secretions in free as well as in IgA- or IgM-bound forms. Therefore, SC is found not only as a membrane receptor but also as a secreted glycoprotein. The receptor (SC) remains covalently linked to its ligand (polymeric IgA) and is not recycled. Hence, it is often called a "sacrificial receptor" (Kühn and Kraehenbuhl, 1982; Solari and Kraehenbuhl, 1985). The fate of the intracellular tail of membrane SC has not been determined but it appears to be intracellularly degraded. Furthermore, it is probable that SC bound to polymeric IgA confers some functional advantages to S-IgA molecules such as increased resistance to proteolytic enzymes (Lindh, 1975).

D. Hepatocytes

The importance of the liver in IgA metabolism has been considered in many studies. Increased levels of serum IgA were seen in patients with liver diseases, particularly with liver cirrhosis (Tomasi and Tisdale, 1964; Kutteh *et al.*, 1982a; Delacroix *et al.*, 1983a; Newkirk *et al.*, 1983) and in animals after carbon tetrachloride poisoning or bile duct ligation (Lemaitre-Coelho *et al.*, 1978a; Kaartinen, 1978). Removal of the ligature led to the prompt return to physiological levels of serum IgA. Vaerman *et al.* (1978) proposed that in rodents, liver functions as an "IgA pump" that regulates serum levels of IgA by transporting circulating polymeric IgA into the bile. By this mechanism significant quantities of IgA are delivered into the intestinal tract (Jackson *et al.*, 1978; Lemaitre-Coelho *et al.*, 1978b; Orlans *et al.*, 1978, 1979; Hall *et al.*, 1980; Reynolds *et al.*, 1980). Molecular and cellular mechanisms of their transport have been elucidated by several investigators. SC present on the surface of hepatocytes was identified as a receptor for polymeric IgA (Orlans *et al.*, 1979; Socken *et al.*, 1979) which consti-

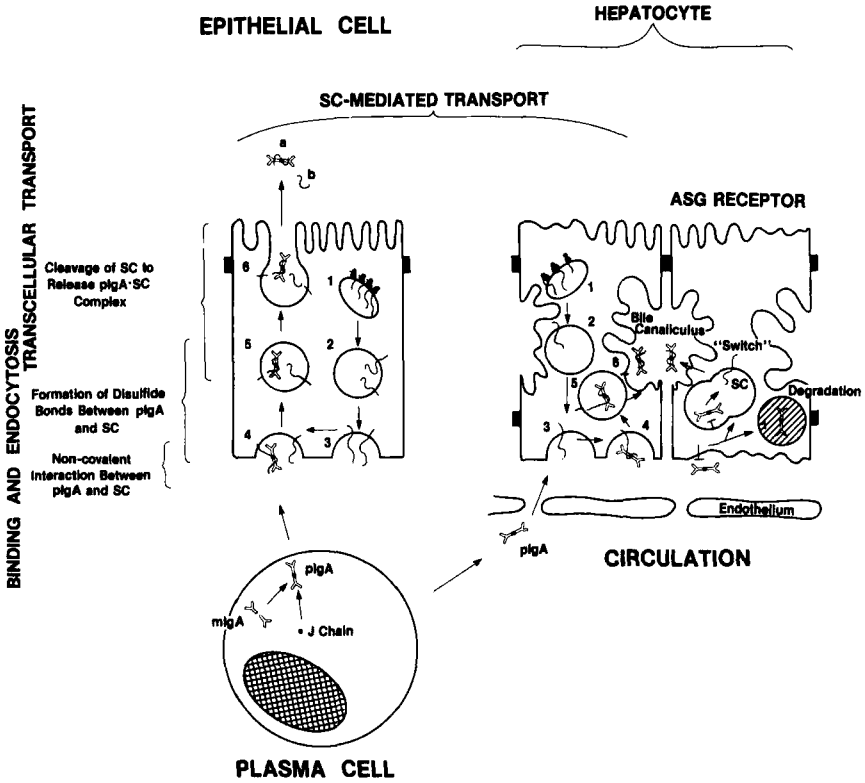

FIG. 4. Model of interactions of IgA with its receptors on epithelial cells and hepatocytes [SC and ASG (asialoglycoprotein) receptor]. SC-mediated interaction results in selective transport of polymeric IgA into various external secretions (milk, saliva, tears, and genitourinary, respiratory, and gastrointestinal fluids) of many species and biliary IgA in rabbits, rats, and mice. Interactions of IgA with the ASG receptor usually result in degradation of internalized IgA (shaded area). Polymeric IgA is assembled in plasma cells from IgA monomers and J chain. SC is produced in rough endoplasmic reticulum as a core-glycosylated protein (1). Glycosylation is completed in the Golgi complex (2) and SC-containing vesicles fuse with the basolateral membrane (3). Polymeric J chain-containing IgA binds to surface membrane SC (4) and the complex is internalized in endoplasmic vesicles (5). Probably at this stage polymeric IgA–SC complex is stabilized by disulfide bridges and SC is enzymatically cleaved to release completed S-IgA molecule. Endoplasmic vesicles fuse with the apical membrane (6) and S-IgA is released (a). Not all of membrane SC is bound to polymeric IgA and free SC (b) can be detected in external secretions. In addition to SC found on surfaces of hepatocytes of some species, the ASG receptor (⊣) can also bind IgA molecules. ASG receptor-bound IgA is intracellularly degraded by lysosome-associated enzymes. Schiff *et al.* (1986b) proposed that under certain conditions polymeric IgA initially bound to the ASG receptor may switch the receptor (to SC) and be secreted as S-IgA. For details see Section III,C and D.

tutes in several species, including rodents and lagomorphs, the primary form of circulating IgA (Vaerman, 1973). SC has been identified in various structural, biochemical, and functional studies as the most important receptor for polymeric IgA that is responsible for the selective and efficient transport of circulating IgA into the bile. For example, it has been shown that in rats and rabbits and to a lesser degree in mice large amounts of IgA are promptly removed from the circulation and transported into the bile (for rats 38.2 and for mice 8.8 mg IgA/kg/day) (Delacroix *et al.*, 1985). The essential role of SC was demonstrated in elegant experiments in which injected polymeric IgA or IgG molecules with an anti-SC activity were selectively transported by SC into the rat bile (Fisher *et al.*, 1979; Lemaitre-Coelho *et al.*, 1981). Hepatic biosynthesis of SC has been studied in rabbits by Mostov *et al.* (1980, 1984), Kühn and Krahenbuhl (1981), Kühn *et al.* (1983), and in rats by Sztul *et al.* (1983, 1985a,b). SC is synthesized as a core-glycosylated transmembrane glycoprotein on polysomes attached to the rough endoplasmic reticulum membrane. Terminal glycosylation occurs following transfer to the Golgi. SC is subsequently routed to the hepatocyte sinusoidal plasma membrane where binding of polymeric IgA takes place. Vesicular carriers that move SC from the Golgi complex to the plasma membrane have not been identified (Sztul *et al.*, 1985b).

After immunoprecipitation with anti-SC, cell-free translation of mRNA from rabbit liver yielded products with molecular weights considerably larger than free forms of SC. Moreover, analyses of membrane forms of both rabbit and rat hepatic SC revealed variations with respect to the molecular weights. One family of molecules had an apparent molecular weight of 116,000 and 120,000 (upper doublet) and the second 91,000 and 95,000 (lower doublet). There was an extensive structural homology between both families as demonstrated by peptide mapping. The small differences in the molecular weights in each family appear to be due to the degree of glycosylation. Although structurally related, high- and low-molecular-weight families differ in the length of the polypeptide chain: in the low-molecular-weight family there is an intramolecular deletion in the N-terminal half of SC which is responsible for a difference of 25,000 Da (Kühn *et al.*, 1983).

Circulating polymeric IgA gains access to the SC on the sinusoidal surface of hepatocytes through openings between endothelial cells which permit molecules with molecular weight less than 1,000,000 to enter the space of Disse. The interaction between polymeric IgA and

hepatocytes and the subsequent processing of completed IgA has been studied by immunofluorescence, immunoelectron microscopy, and autoradiography using perfused liver, dispersed hepatocytes, or monolayer cultures of hepatocytes (Hopf *et al.*, 1978; Mullock *et al.*, 1979, 1980a,b, 1983; Birbeck *et al.*, 1979; Renston *et al.*, 1980; Wilson *et al.*, 1980; Tolleshaug *et al.*, 1981; Jones *et al.*, 1982; Limet *et al.*, 1982a,b; Takahashi *et al.*, 1982; Gebhardt, 1983, 1984; Goldman *et al.*, 1983; Delacroix *et al.*, 1984; Hoppe *et al.*, 1985; Solari *et al.*, 1986). Various preparations of polymeric but not monomeric IgA bind to the surfaces of hepatocytes and are subsequently internalized and transported in the form of vesicles with a diameter of 100–140 nm to the bile cannaliculi where the content is discharged into the bile by exocytosis (Fig. 4). The IgA-containing vesicles do not fuse with lysosomes and IgA discharged into the bile is found in intact SC-associated form. As in the case of epithelial cells, the site of the cleavage of SC with subsequent release of polymeric IgA complexed to the membrane form of SC and the specific proteolytic enzymes(s) involved have not been determined.

IgA interactions with hepatocytes can also be mediated through hepatic receptors specific for asialoglycoproteins (hepatic binding protein, HBP) (Fig. 4). Stockert *et al.* (1982) demonstrated by inhibition of receptor-mediated erythroagglutination that sera of patients with high levels of IgA or IgA_1 but not IgA_2 myeloma strongly inhibited this reaction. Furthermore, these authors showed that the recognition site appears to be localized in the hinge region of IgA_1 proteins which is rich in O-glycosidically linked oligosaccharide side chains (see above). Indeed, the oxidation of the galactose or *N*-acetylgalactosamine residues of IgA_1 by galactose oxidase abolished the inhibition of receptor-mediated erythroagglutination. Various human IgA proteins and HBP isolated from rat liver were used in these experiments. Polymeric IgA_1-mediated inhibition was at least an order of magnitude greater than monomeric IgA of the same subclass. The presence and properties of HBP have been described on hepatocytes from several species (Ashwell and Morrell, 1974; Ashwell and Harford, 1982; Schwartz, 1984). The HBP recognizes terminal galactose residues of desialylated glycoproteins and Stockert *et al.* (1982) proposed that HBP as well as SC can play an important role in the endocytosis of IgA by hepatocytes.

This point was also addressed in a mouse model of transport of IgA-containing immune complexes. Although polymeric mouse myeloma IgA protein (MOPC 315), specific for the dinitrophenyl (DNP) hapten linked to nonglycosylated carrier, mediated a selective transport of

such immune complexes from circulation into the bile, this process was not inhibited by the presence of asialofetuin (Brown *et al.*, 1982). The hepatobiliary transport of intact human polymeric myeloma IgA proteins in a mouse model was also not inhibitable by asialofetuin (Phillips *et al.*, 1984). However, desialyation of human polymeric IgA resulted in its rapid clearance from the circulation and subsequent deposition into the liver in a manner similar to other desialyated glycoproteins. Small amounts of the desialyated polymeric IgA appeared in bile as an undigested molecule. To achieve a highly significant inhibition of the hepatobiliary transport of desialyated human polymeric IgA, both asialofetuin and the intact IgA molecule (presumably transported by an SC-dependent mechanism) were required. Thus, the asialoglycoprotein receptor may indeed represent an additional mechanism for the transport of desialyated polymeric IgA from circulation into bile.

The importance of HBP in the carbohydrate-mediated serum clearance and hepatobiliary transport of IgA was demonstrated by Tomana *et al.* (1985). Human S-IgA which contains SC was rapidly cleared from the circulation by the liver. This clearance was inhibited by prior injection of asialofetuin (but not mouse polymeric IgA), suggesting that S-IgA uptake is mediated by HBP. This conclusion was strengthened by *in vitro* studies with the particulate fraction (pellet of liver homogenate) or the plasma membrane fraction of mouse hepatocytes: S-IgA inhibited binding of asialoorosomucoid used as a probe for HBP. Because of its high carbohydrate content and the presence of terminal galactose residues in the molecule (Purkayastha *et al.*, 1979; Mizoguchi *et al.*, 1982), SC and the hinge peptide from IgA_1 were the most potent ligands that inhibited binding of asialoorosomucoid to HBP.

Various ligands bound to their corresponding receptors on hepatocytes are subsequently processed through specific pathways. Differential uptake of polymeric IgA through the SC receptor, and asialoglycoproteins through HBP, were studied in a rat model by Underdown *et al.* (1983) and Schiff *et al.* (1984, 1986a). Polymeric IgA bound to SC is quantitatively transported through the hepatocyte in the form of small vesicles into the bile; IgA remains undigested and IgA-containing vesicles do not fuse with enzyme-filled lysosomes. In contrast, asialoglycoproteins which interact with HBP are processed mostly by a degradative pathway: vesicles that contain ligand–receptor complexes fuse with lysosomes resulting in degradation of the ligand (Fig. 4). Schiff *et al.* (1984) suggested that ligands destined for different compartments in hepatocytes are endocytosed into different vesicles.

Therefore, the sorting should occur at the cell membrane because the receptors for the two pathways are rarely in juxtaposition. Electron microscopic autoradiography provided further support for this possibility. This concept, however, is at variance with results from other laboratories. Courtoy *et al.* (1984) demonstrated that galactosylated bovine serum albumin and polymeric IgA were endocytosed within the same vesicle. Furthermore, Geuze *et al.* (1984) demonstrated the presence of membrane SC, HBP, and another receptor (mannose-6-phosphate) within the same coated pits and vesicles as well as in the compartment of uncoupling of receptors and ligands where sorting of the different receptors to their various destinations occurs. To resolve this controversy, Schiff *et al.* (1986a) used a rat liver perfusion model and human polymeric IgA as a heterovalent ligand capable of binding to both HBP and SC. The authors proposed that human polymeric IgA, though initially captured by HBP, is transported by an SC-dependent mechanism in the hepatocyte. Thus, there appears to be switching of the ligand that binds to two different receptors during the transport. Although there may be certain species differences with respect to the handling of human IgA, these results are at variance with the processing of intact or desialyated human IgA of both subclasses and molecular forms by the mouse liver (Phillips *et al.*, 1984): asialofetuin did not significantly influence hepatobiliary transport of intact human polymeric IgA. In contrast, polymeric mouse myeloma IgA proteins (which are bound by SC but not by HBP) strongly inhibited appearance of human IgA in mouse bile.

Further experiments with, for example, human S-IgA (in which SC is covalently linked to polymeric IgA) or conjugates of polymeric IgA with asialoglycoprotein, or desialyated human polymeric IgA, will be necessary to confirm the hypothesis of receptor switching during the transport of IgA through hepatocytes.

The existence of an additional IgA receptor on rat hepatocytes, unrelated to SC or HBP, has been considered by Tolleshaug *et al.* (1981), who maintained that this receptor bound human polymeric (including S-IgA) as well as monomeric IgA. Because desialyation of IgA with neuraminidase did not increase IgA binding and the addition of EGTA (which interferes with Ca^{2+}-dependent binding to HBP) resulted in only 44% inhibition, the authors claimed that an asialoglycoprotein receptor is not involved. However, the conditions used for desialyation were not optimal for the loss of sialic acid.

Interaction between various forms of IgA and hepatocytes are strongly species dependent (Vaerman *et al.*, 1982; Orlans *et al.*, 1983). Although HBP has been found on hepatocytes of many species, SC is

present on rat, rabbit, and mouse hepatocytes (see above), but not, it appears, on human cells. Initial reports of the occurrence of SC on human hepatocytes (Hsu and Hsu, 1980; Foss-Bowman *et al.*, 1983) were not confirmed by others who used a broad variety of immunochemical and immunohistochemical techniques (Hopf *et al.*, 1978; Nagura *et al.*, 1981; Delacroix and Vaerman, 1983; Chandy *et al.*, 1983; Brandtzaeg, 1985).

The interaction of IgA with receptors on hepatocytes is of considerable functional significance. In several species the hepatobiliary transport of polymeric IgA reinforces intestinal immunity. For example, it has been estimated that in rats the largest part of S-IgA found in the upper jejunum is derived from the bile rather than from local synthesis in the intestine (Lemaitre-Coelho *et al.*, 1978b). In rats and rabbits the liver clears daily *24 times the entire plasma pool of polymeric IgA* (Delacroix *et al.*, 1985).

SC-mediated hepatobiliary transport is not solely restricted to free polymeric IgA: small immune complexes (molecular weight less than 1,000,000) are also transported from the circulation into the bile (Peppard *et al.*, 1981; Russell *et al.*, 1981; Socken *et al.*, 1981a,b). Such transport of preformed immune complexes containing pIgA with anti-DNP activity and DNP on protein carrier was demonstrated in mice and rats (Russell *et al.*, 1981; Socken *et al.*, 1981a,b). Furthermore, intravenously injected bacterial antigens, such as pneumococcal type III capsular polysaccharide and C carbohydrate as well as phosphorylcholine conjugated to human serum albumin, were selectively transported into the bile by passive immunization with minute amounts of monoclonal IgA but not IgM or IgG antibodies to these antigens (Russell *et al.*, 1983). Orally administered DNP-labeled human serum albumin, which is absorbed in small quantities and is detectable in the circulation, was also effectively eliminated into the bile by intravenously injected polymeric IgA with anti-DNP activity (Brown *et al.*, 1984). Therefore, results suggest that the continual process of SC-dependent hepatic uptake of circulating polymeric IgA and IgA-containing immune complexes may represent a natural pathway of disposal of absorbed antigens by a noninflammatory means. It was operational in animals that had been depleted of the C3 complement component and that had at least a partially blocked reticuloendothelial system (Brown *et al.*, 1982).

Under natural conditions a humoral immune response is not restricted to a single isotype and immune complexes may contain, in addition to IgA, complement-binding and opsonizing antibodies. The fate of such mixed immune complexes was investigated by Peppard *et*

al. (1981) and Phillips *et al.* (1986). Polymeric IgA complexed to an anti-idiotype of the IgG class appeared to play a dominant role with respect to the fate of such immune complexes found in the bile.

In other species, including dogs, guinea pigs, and humans, circulating IgA is not transported in large quantities into the bile probably because of the absence of SC on the surface of hepatocytes (Dooley *et al.*, 1982; Delacroix and Vaerman, 1983; Delacroix *et al.*, 1982a, 1983a,b, 1984). For example, only approximately half of the polymeric IgA found in human hepatic bile is derived from plasma (Delacroix *et al.*, 1982a); the other half appears to be synthesized locally in the liver by IgA- and SC-producing plasma and epithelial cells that are organized into minor glands embedded in or adjacent to the wall of large hepatic ducts (Nagura *et al.*, 1981, 1983). Molecular analyses of IgA from hepatic bile revealed that in addition to typical S-IgA molecules, half of the polymeric IgA in bile is not associated with SC but contains J chain (Kutteh *et al.*, 1982a; Delacroix *et al.*, 1982a; Delacroix and Vaerman, 1983). It is unclear whether this polymeric IgA reached the bile by an alternative mechanism or whether it represents IgA that was captured from the circulation by HBP but escaped the degradation pathway.

The liver also appears to be involved in regular catabolism of immunoglobulins (Fukumoto and Brandon, 1982), including both polymeric and monomeric IgA (Delacroix, 1985). In man only negligible amounts (approximately 1 mg/kg/day) of the total IgA produced in the bone marrow, spleen, and lymph nodes (approximately 20 mg/kg/day) reaches external secretions (Delacroix, 1985). Therefore, most of the IgA from the circulating pool is internally catabolized. It appears that in mice the liver is the primary site of IgA catabolism; kidneys and the skin may also be involved (Moldoveanu *et al.*, 1985b). Sites and recognition mechanisms involved in catabolism of human IgA are unknown. These considerations are summarized in Fig. 5.

IV. Cellular Interactions in the IgA Biosynthesis and Immune Response

A. Cellular and Molecular Aspects of IgA Biosynthesis

IgA-producing plasma cells are distributed in various lymphoid and nonlymphoid tissues and are particularly preponderant in the lamina propria of the gut; in salivary, lacrimal, and lactating mammary glands; and in the human bone marrow (Heremans, 1974; Lamm, 1976; Brandtzaeg and Baklien, 1976; Brandtzaeg, 1985). It is surprising that the latter tissue is so rarely considered as the major source of

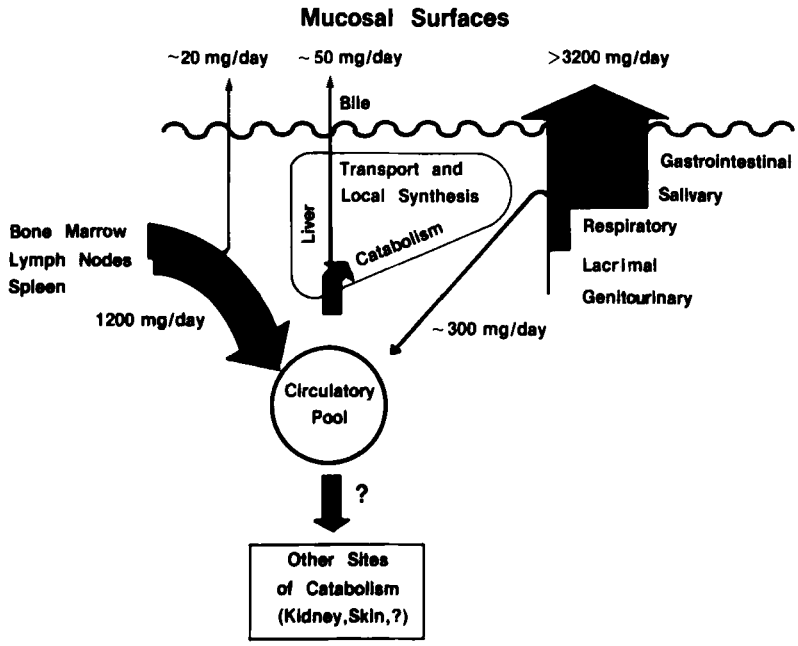

FIG. 5. Estimated contributions of systemic (bone marrow, lymph nodes, and spleen) and mucosa-associated compartments of the IgA system to mucosal secretions and the circulatory pool. A large amount of predominantly monomeric IgA produced in bone marrow is catabolized in the liver and other sites; in humans IgA from the circulation contributes little to the pool of IgA found on mucosal surfaces. Most IgA produced at secretory sites is selectively transported by an SC-dependent pathway into external secretions; approximately 10% enters the circulatory pool. This diagram is based on results summarized in Delacroix (1985) and others. For details see Section III,D.

circulatory IgA (Hijmans *et al.*, 1971; McMillan *et al.*, 1972; Benner *et al.*, 1981; Kutteh *et al.*, 1982b; Alley *et al.*, 1982; MacDermott *et al.*, 1983; Mestecky *et al.*, 1986b).

As described above, IgA occurs in different body fluids in predominantly polymeric (external secretions) or monomeric (plasma, cerebrospinal fluid) forms with a characteristic distribution of IgA_1 and IgA_2 molecules.

It was initially proposed that polymeric S-IgA was assembled from monomeric IgA by complexing with SC after its secretion from plasma cells (Tomasi *et al.*, 1965; South *et al.*, 1966). However, subsequent immunochemical studies of rabbit (Lawton and Mage, 1969) and human (Bienenstock and Strauss, 1970) S-IgA molecules convincingly showed that polymers are formed within IgA-producing cells *before* their selective transport into external secretions. The tissue distribu-

tion of cells that produce polymeric or monomeric IgA has been determined by immunochemical analyses of IgA produced *ex vivo* by various tissues. Tissue culture supernatants of salivary glands (Hurliman and Zuber, 1968), gut mucosa (Kutteh *et al.*, 1982b; MacDermott *et al.*, 1983), or perfusates of intestinal segments (Bull *et al.*, 1971) contained predominantly polymeric forms of IgA, while monomeric IgA was more abundant in tissue culture media of human bone marrow, spleen, and certain lymph nodes (Kutteh *et al.*, 1982b; Alley *et al.*, 1982). An alternative approach used for the identification of cells producing polymeric or monomeric IgA is based on the presence of intracellular J chain in cells that produce polymers and the ability of such cells to bind SC (Brandtzaeg, 1973). The rationale is based on the earlier *in vitro* observations that J chain is linked extracellularly only to polymeric IgA and IgM and that both of these immunoglobulins in their polymeric but not monomeric forms are capable of binding SC (Mach, 1970; Radl *et al.*, 1971; Weicker and Underdown, 1975; Lindh and Bjork, 1976a,b; Brandtzaeg, 1977). Brandtzaeg (1973, 1976c, 1983) and others (Radl *et al.*, 1974; Crago and Mestecky, 1979; Moldoveanu *et al.*, 1984) exploited these findings in extensive immunohistochemical examinations of tissue sections, cell suspensions from various human tisues, and human lymphoblastoid cell lines. Under normal conditions an absolute majority of IgA-containing cells in secretory glands and tissues also contain J chain whereas such cells in, for example, normal bone marrow do not (Kutteh, 1982b; Brandtzaeg, 1983). Staining with fluorochrome-labeled anti-J chain is further enhanced by the pretreatment of alcohol-fixed tissue sections with acid urea, which leads to the exposure of masked antigenic determinants of intracellular J chain (Brandtzaeg, 1983). Although J chain can be occasionally detected in cells that produce monomeric IgG or IgD (Brandtzaeg, 1983; Brandtzaeg *et al.*, 1979; Mestecky *et al.*, 1977, 1980a), it is claimed that treatment with acid urea does not increase the intensity of immunofluorescence (Brandtzaeg, 1983). SC binding tests performed on mildly fixed tissue sections or cell suspensions (extensive fixation and acid urea abrogrates SC binding) revealed that the majority of IgA-containing cells in secretory glands and tissues but not those in extraglandular tissues bind SC (Brandtzaeg, 1983). These findings imply that polymeric IgA-secreting cells contain enough intracellularly assembled polymers to bind SC. This is quite surprising in view of biochemical analyses of the molecular forms of intracellular IgA. In earlier studies of the biosynthetic pathways of polymeric immunoglobulins, Parkhouse (1971), Parkhouse and Askonas (1969), and Bargelesi *et al.* (1972) demonstrated in mouse cells secreting poly-

meric IgA or IgM that a majority of intracellular immunoglobulins are present as monomers and proposed that polymerization occurs shortly before or at the time of secretion. Others (Tartakoff *et al.*, 1979) have found considerable amounts of intracellular polymeric IgM in lysates of cultured mouse cells. In contrast, intracellular forms and pathways of assembly of IgA in human cells have not been studied extensively by biochemical methods. Buxbaum *et al.* (1974) compared secreted and intracellular forms produced by plasma cells from the bone marrow of IgA myeloma patients. Although 20–50% of secreted IgA occurred in polymeric form, measurable quantities of intracellular polymeric IgA were detected only in one of the nine samples examined. Kutteh *et al.* (1982b) compared molecular forms of intracellular and secreted IgA from cell suspensions of various human tissues kept in culture. Cell lysates from bone marrow, spleen, lymph nodes, and tonsils contained predominantly monomeric IgA despite the fact that tonsils and lymph nodes secreted considerable amounts of polymeric IgA into the culture supernatants. Lysates and tissue culture supernatants of human ileum both contained predominantly polymeric IgA, most probably due to the significant contamination with epithelial cells that released preformed polymeric S-IgA. In subsequent studies, Moldoveanu *et al.* (1984) compared secreted and intracellular forms of IgA from pokeweed mitogen (PWM)-stimulated peripheral blood lymphocytes from normal donors and an Epstein–Barr virus (EBV)-transformed human cell line using molecular sieve chromatography, electrophoresis in sodium dodecyl sulfate, and sucrose density ultracentrifugation. The absolute majority of intracellular IgA occurred in a monomeric form even when the predominant form of secreted IgA was polymeric. However, small amounts of polymeric IgA, capable of *in vitro* SC binding, were detected in some cell lysates. It appears that these low amounts of intracellular polymeric IgA are responsible for SC binding in the immunofluorescence test. The predominance of intracellular monomers in polymeric IgA-secreting cells suggests that the pathway of assembly of human IgA molecules is analogous to that described for mouse IgA synthesis. It should be kept in mind that the basic question, whether one IgA plasma cell secretes *only* polymeric or monomeric IgA or both forms concurrently or sequentially, has not been completely answered.

The role of J chain in the process of intracellular polymerization has not been conclusively established. Because (1) J chain is preferentially expressed in cells that produce polymeric immunoglobulins (Halpern and Coffman, 1972; Parkhouse, 1972; Mather *et al.*, 1981), (2) secreted polymers are associated with J chain, and (3) a small

proportion of intracellular immunoglobulin is linked to J chain, it has been proposed that this chain initiates polymerization by binding first to a monomeric molecule of intracellular IgA or IgM which is subsequently linked to another monomer, thus forming a dimeric molecule (Chapuis and Koshland, 1974, 1975; Hauptman and Tomasi, 1975). These considerations have been extensively reviewed (Inman and Mestecky, 1974; Koshland, 1975, 1985). Whether J chain indeed *initiates* polymerization is questionable. Stott (1976) observed that intracellular IgM may occur in lysates of certain mouse cell lines in a tetrameric form that lacks J chain. This polypeptide, linked to monomeric IgM, was added as a last step in the process of polymerization. The discrepancies in the above-cited studies concerning the *in vivo* role of J chain in the process of polymerization may also reflect differences in the assembly of polymeric immunoglobulins which could be unique for the lymphoid cell lines used. Furthermore, *in vitro* studies of polymerization and the role of J chain in this process provided contradictory results (Kownatzki, 1973; Eskeland, 1974; Della Corte and Parkhouse, 1973; Parkhouse and Della Corte, 1973, 1974), most probably due to the differences in experimental conditions and the use of J chain isolated by procedures that may have led to various degrees of denaturation.

One of the most puzzling questions concerning the role of J chain is its expression in human cells at their earliest differentiation stages and in cells that produce monomeric immunoglobulins of the IgG and IgD isotypes. Using immunochemical and immunohistochemical techniques, J chain has been detected in several, but not all, human leukemic cells and lymphoblastoid cell lines that were phenotypically classified as null, pre-B, or B cells (McCune *et al.*, 1981; Hajdu *et al.*, 1983; Max and Korsmeyer, 1985). Particularly intriguing is the finding that J chain can be expressed in cells that lack other immunoglobulin chains (Mason and Stein, 1981; McCune *et al.*, 1981; Hajdu *et al.*, 1983). At present, it is unclear whether such an early expression of J chain occurs only in some malignant cells and lymphoblastoid cell lines or whether it occurs regularly during differentiation of normal cells of the B lymphocyte lineage.

The presence of J chain in malignant or normal cells that produce monomeric IgG, IgD, or only H or L chains (Brandtzaeg, 1974b, 1976a; Radl *et al.*, 1974; Kaji and Parkhouse, 1974, 1975; Mestecky *et al.*, 1977, 1980a; Sarasombath *et al.*, 1977; Brandtzaeg *et al.*, 1979; Laurent *et al.*, 1981; Bast *et al.*, 1981) remains unexplained. Brandtzaeg (1985) and Brandtzaeg and Korsrud (1984) proposed that J chain expression in normal IgG and IgD cells may be a sign of clonal matu-

ration: relatively immature cells that are found in glandular tissues and in tonsillar intrafollicular areas express J chain with greater frequency than such cells in nonglandular tissues and extrafollicular areas. The possibility that the synthesis of J chain in cells that produce monomeric immunoglobulin is a remnant of preceding synthesis of polymeric IgM is unlikely. As shown by immunofluorescence of normal tissues or mitogen-stimulated peripheral blood lymphocytes, cells that are positive (by cytoplasmic straining) for two isotypes are encountered infrequently. In the PWM system the proportion of IgM-, IgG-, and IgA-positive cells remains relatively constant throughout the entire culture period despite the remarkable increase in cells positive for cytoplasmic immunoglobulins that occurs on days 4–6 of culture (Mestecky *et al.*, 1977).

In addition to the characteristic distribution of plasma cells that secrete polymeric or monomeric forms of IgA, cells that produce IgA_1 and IgA_2 subclasses display a unique distribution in various lymphoid organs and tissues (for review see Mestecky and Russell, 1986). In general, with the exception of the large intestine, where IgA_2-containing cells are more frequent, IgA_1 cells are predominant to varying degrees in all other glandular and secretory tissues (Crago *et al.*, 1984; Kett *et al.*, 1986). The predominance of IgA_1 cells is particularly striking in the bone marrow (Skvaril and Morell, 1974; Crago *et al.*, 1984), spleen, tonsils, and some lymph nodes.

Although most investigators have observed this characteristic distribution of IgA_1 and IgA_2-containing cells, differences in distribution were seen in certain tissues. For example, Andre *et al.* (1978) observed more IgA_1 cells in the large intestine, whereas Crago *et al.* (1984) and Kett *et al.* (1986) did not. Although IgA_1 cells were more frequent in lacrimal glands in all studies, this predominance was more pronounced in tissues examined by Kett *et al.* (1986) than in tissues examined by Allansmith *et al.* (1985). These differences may be partly due to the use of polyclonal (Andre *et al.*, 1978) vs monoclonal antibodies (Crago *et al.*, 1984; Allansmith *et al.*, 1985; Kett *et al.*, 1986), tissue processing, staining procedures, and large variations in the proportions of IgA_1 and IgA_2 cells in the same tissue type obtained from various individuals.

The reason for the selective distribution of IgA_1 and IgA_2 cells, and particularly a preponderance of IgA_2 cells in the large intestine, is at present unclear. One may speculate that local stimulation with antigens that induce a predominant immune response in one of the IgA subclasses may lead to the expansion of a certain subclass in that tissue. For example, S-IgA antibodies to endotoxin of gram-negative

bacteria are predominantly of the IgA_2 subclass (Brown and Mestecky, 1985). Large intestine, the locale of most intensive stimulation by endotoxin, displays more IgA_2 than IgA_1 cells (Crago *et al.*, 1984; Kett *et al.*, 1986).

The proportion of IgA_1- to IgA_2-positive cells approximates the IgA_1 to IgA_2 ratio in a given secretion (Delacroix, 1985). Unfortunately, precise comparative studies of the proportion of IgA_1 and IgA_2 cells and S-IgA molecules in the gland and corresponding secretion have not been performed with the use of identical subclass-specific reagents. Therefore, there is an apparent discrepancy in the proportion of IgA_1 (59%) and IgA_2 (41%) in tears (Delacroix *et al.*, 1982b) and in cells of the lacrimal gland (81% and 19%, respectively) (Kett *et al.*, 1986). It is possible that polymeric IgA_2 may be more readily transported into external secretions than IgA_1 polymers but no experiments have been performed that address this point.

B. Induction of IgA Immune Response

The protective role of S-IgA has been demonstrated in several experimental systems. S-IgA can neutralize biologically active antigens such as viruses, toxins, and enzymes, prevent uptake of antigens from the intestinal tract, inhibit adherence of bacteria to epithelial surfaces, and enhance the antibacterial effects of innate immune factors (for review see Heremans, 1974; Bienenstock and Befus, 1980; Hanson *et al.*, 1980; Underdown and Schiff, 1986). Therefore, the selective induction of specific S-IgA antibodies is desirable for the prevention of infectious diseases contracted through the large area of mucosal surfaces and for inhibition of the uptake of potential allergenic environmental substances (Walker and Isselbacher, 1976). Early immunization attempts indicated that an antigen applied to the mucosal membranes penetrates into deeper layers and stimulates differentiation of resident B lymphocytes into IgA plasma cells that produce antibodies specific to the applied antigen. The IgA antibodies produced are then actively transported through the epithelial cells on the mucosal surfaces (for review see Mestecky *et al.*, 1978a). Numerous examples demonstrate the efficiency of this pathway of immunization. The application of antigens through the conjunctival sac of one eye led to the appearance of antibodies in tears from the immunized site but not in secretions from the other eye (Centifanto *et al.*, 1970). Similarly, the application of viral antigen (inactivated poliovirus vaccine) into a segment of large intestine (Ogra and Karzon, 1969) or vagina (Ogra and Ogra, 1973) resulted in the appearance of a poliovirus-specific S-IgA response confined to the site of immunization

without a concomitant response in nonimmunized areas. Furthermore, retrograde instillation or injection of antigens into parotid or mammary glands (Genco and Taubman, 1969; Emmings *et al.*, 1975; McGhee *et al.*, 1975; Hurlimann and Lichaa, 1976) or repeated topical application to oral mucosa (Nair and Schroeder, 1983) led to the appearance of specific antibodies in secretions of the immunized gland and detection of plasma cells producing antibodies in the glandular stroma. These are only a few examples of experiments demonstrating that a site-restricted IgA-associated response can be induced by local stimulation of mucosal membranes and secretory glands with antigens. In some cases a concomitant systemic response may be induced which is manifested by the presence of serum antibodies of other Ig isotypes (Hurlimann and Lichaa, 1976). Both systemic immunization and oral ingestion of antigens of short duration usually do not lead to the induction of a preferential serum IgA response (for review see Heremans, 1974, Table XVI); instead, most of the circulatory antibodies are present in IgM and IgG classes. However, a prolonged oral exposure to protein (ferritin) or particulate (sheep erythrocytes) antigens in mice may induce serum IgA antibodies (Crabbe *et al.*, 1969; Heremans and Bazin, 1971). Recently Alley *et al.* (1986) have demonstrated that prolonged oral immunization of mice with sheep erythocytes results in the appearance of specific IgA-producing cells in the bone marrow.

IgA, IgM, and IgG antibodies specific for many microbial and food antigens have been detected in sera of normal individuals (Russell *et al.*, 1986; Mestecky and Russell, 1986). They were associated mostly with the IgA_1 subclass and occurred in both polymeric and monomeric IgA forms with considerable individual variability. After prolonged exposure to certain viral antigens, serum IgA antibodies shift from the predominantly polymeric form at the onset to the monomeric form in the later period after exposure to the antigen (Brown *et al.*, 1985, 1987; Negro-Ponzi *et al.*, 1985). This shift in the pattern of the serum IgA response suggests that such antibodies may be produced at mucosal sites at the beginning, but later the bone marrow may be the principal site of monomeric serum IgA antibodies to at least some environmental antigens.

Local stimulation with antigen is not the only pathway that leads to an effective induction of a secretory immune response. Secretions of glands that are anatomically remote from the site of immunization such as mammary, salivary, and lacrimal glands contain S-IgA antibodies to antigens encountered through the respiratory or gastrointestinal tracts. Extensive studies in several laboratories have been con-

ducted that led to the proposal of a common mucosal immune system as described below (Section IV,D). Specialized lymphoid tissues associated with mucosal surfaces play an essential role in the induction and regulation of generalized immune responses in external secretions. A discussion of the unique origin and properties of cells involved is pertinent at this point.

C. Mucosa-Associated Lymphoid Tissues

In most mammalian species, discrete lymphoid follicles are found along the wall of the small intestine, and these are collectively termed the gut-associated lymphoreticular tissue or GALT. The GALT is remarkably well developed in rabbits where it represents greater than 70% of the animals' total lymphoid tissue. GALT includes (1) the Peyer's patches, which are distinct nodules mainly along the small intestine, and (2) the appendix, a lymphoid organ in the rabbit which exceeds the size of the spleen. The rabbit also possesses the *Sacculus rotundus*, an overdeveloped, terminal Peyer's patch at the ileocaecal junction. Mammals also contain single follicles of lymphoid cells throughout the small intestine and colon; these are termed solitary lymphoid nodules (SLN). Although each of these tissues, e.g., Peyer's patches, and the appendix (and to a lesser extent the SLN), exhibit anatomical similarities, including a dome region with a covering specialized epithelium that contains lymphoreticular cells, important differences also exist. For example, the appendix has a less prominent parafollicular region or T cell-dependent area (TDA) when compared with the PP, and isolated lymphocytes from the appendix exhibit greater relative numbers of surface immunoglobulin-positive (sIg^+) cells (B cells).

Generally, most studies with isolated GALT cells have been performed with PP, with the assumption that PP are a major component of GALT. Therefore, we will limit this discussion to the PP and the reader is referred to fuller descriptions of the appendix and other GALT sites (Hanaoka and Waksman, 1970; Watanabe and Tashiro, 1971; Bockman and Cooper, 1973; Blythman and Waksman, 1973; Parrott, 1976; Waksman and Ozer, 1976; Bockman, 1983).

1. Functional Anatomy of the Peyer's Patch

Peyer's patches are named for their discoverer, the anatomist J. C. Peyer, who first described this tissue in humans in 1677. Interestingly, human PP are much less distinct than those seen in most experimental animal species, and because of their inaccessibility and the resultant difficulty of visualizing them, human PP have remained

poorly characterized. For many years, most textbooks suggested that only 20 to 30 PP occurred in the human intestine. However, in a now classic study, Cornes (1965) showed that during gestation, 45–100 PP occur and their numbers reach a maximum at puberty (~240) and decline slowly with age. Thus, PP clearly represent a major lymphoid tissue in the human gut. Most extensive studies of lymphoreticular cells isolated from PP have been performed in rabbits and mice. In mice, PP are distinct nodules along the small intestine, and one can usually see 8–10 PP in each animal. Nevertheless, all experimental species possess PP, including nonhuman primates (Owen and Jones, 1974), rats (Sminia and Plesch, 1982), swine (Chu *et al.*, 1979), calves (Waksman, 1973), and dogs. It is interesting that PP-like structures have also been described in avian species (Befus *et al.*, 1980).

An important characteristic of the PP is the presence of a unique epithelium which covers the dome region. This epithelium contains cuboidal epithelial cells, reduced numbers of goblet cells, and a specialized antigen-sampling cell, termed a follicular-associated epithelial cell (FAE cell; Bockman and Cooper, 1973) or a microfold cell (M cell; Owen and Jones, 1974). The FAE or M cells are heavily infiltrated with lymphoreticular cells from the dome region, and this unique association has been termed a *lymphoepithelium*. The origin of the M cell is unknown, however, it may develop from the less differentiated cuboidal epithelial cell, perhaps under the influence of closely associated lymphoreticular cells (Smith and Peacock, 1980). M cells exhibit short, irregular microvilli (microfolds; Owen and Jones, 1974), numerous cytoplasmic vesicles, and thin cytoplasmic extensions which surround lymphoreticular cells. Bockman and Cooper (1973) first showed that FAE cells (M cells) actively pinocytose ferritin from the gut lumen and transport this to underlying lymphoid cells. Owen (1977) showed that horseradish peroxidase (HRP), when injected into mouse intestines, selectively adhered to the M cell surface and within minutes the HRP was found in pinocytotic vesicles. Within 1 hour, the HRP was associated with lymphocytes in the dome and also occurred in extracellular spaces between M cells. Uptake of proteins by M cells does not result in lysosomal enzyme degradation, and the intact protein, presumably fully immunogenic, reaches underlying dome lymphoreticular cells (Ducroc *et al.*, 1983). M cells are also actively phagocytic, and allow sampling of particulate lumenal antigens such as viruses (Wolf *et al.*, 1981) and even whole bacteria (Carter and Collins, 1974). Thus the M cell, in the absence of a mucus layer due to a paucity of goblet cells, serves as an antigen-sampling mechanism for the uptake of lumenal antigens and their intact trans-

port across the epithelium. It is possible that M cells act as epithelial antigen-presenting cells; however, this remains to be established. This unique antigen-uptake mechanism by M cells is characteristic of lymphoepithelia associated with GALT and bronchus-associated lymphoreticular tissue (BALT), since these cells have been found in the appendix (Bockman and Cooper, 1973), the caecal Peyer's patch (Owen, 1978), the tonsils (Olah and Everett, 1975), and the BALT (Tenner-Racz *et al.*, 1979; Bienenstock *et al.*, 1973).

Histologically and functionally, the PP can be divided into three major regions: (1) the dome, (2) lymphoid follicles (corona with one to two germinal centers, the B cell zone), and (3) a parafollicular or T-dependent area (TDA, T cell zone). The PP do not contain distinct efferent lymphatics, and cells exit from this tissue via efferent lymphatics which drain into the mesenteric lymph nodes. Classic studies by Gowans and Knight (1964) showed that PP are a major compartment for recirculating lymphocytes, and B and T lymphocytes enter the PP at high endothelial (postcapillary) venules (HEV) in the parafollicular (TDA) region.

Each PP region has a characteristic array of cells. The dome consists largely of lymphocytes, both T and B; however, significant numbers of macrophages which appear functionally active are also present. Underneath the dome region are the follicles which represent the B cell zones. Generally, follicles contain one to two germinal centers which are enriched in B cells committed to IgA (surface IgA positive, $sIgA^+$) (Butcher *et al.*, 1982b), a characteristic feature of GALT. However, unlike germinal centers in other secondary lymphoid tissue, B cell development and differentiation into plasma cells rarely occur in this tissue (Butcher *et al.*, 1982b; Craig and Cebra, 1971; Jones *et al.*, 1974; Rudzik *et al.*, 1975; Cebra *et al.*, 1980; 1983a). Adjacent to the follicles is the so-called parafollicular region which represents the T cell zone, i.e., thymus-dependent area (TDA) (Barg and Draper, 1975; Joel *et al.*, 1971; Joel *et al.*, 1972; Chanana *et al.*, 1973; Ferguson and Parrott, 1972). Both the B cell and T cell zones possess antigen-processing cells which are Ia^+ and exhibit a dendritic cell-like morphology (Sminia and Plesch, 1982).

Unlike the thymus and Bursa-equivalent organs, the PP are poorly developed at birth. However, T lymphocytes recently migrating from the thymus populate the dome and TDA within 1 week (Barg and Draper, 1975; Joel *et al.*, 1971, 1972; Chanana *et al.*, 1973) and T cell zones are completely developed within 2–4 weeks (Ferguson and Parrott, 1972). The B cell zones develop more slowly, and are dependent upon the gut microflora, since germ-free mice have poorly devel-

oped follicles with no germinal centers (Pollard and Sharon, 1970). Likewise, isografts of fetal gut transplanted over kidney capsules remain poorly developed and fail to exhibit germinal center formation (Ferguson and Parrott, 1972).

2. *Studies with Dissociated PP Cells*

Many hypotheses have been expressed concerning the function of PP in respect to immunity. Kagnoff (1975; Kagnoff and Campbell, 1974) has performed extensive studies describing the potential of the PP lymphocytes for responses both *in vivo* and *in vitro*. Murine PP cultures were shown to contain antigen-sensitive T and B cells whereas they lacked sufficient macrophages (Mϕ) for support of *in vitro* immune responses (Kagnoff and Campbell, 1974). Subsequent investigations demonstrated that oral presentation of antigens to mice resulted in the induction of T helper (Th) cells in PP (Kagnoff, 1975). Further studies (Kagnoff *et al.*, 1974) indicated that PP from athymic, nude (*nu/nu*) mice lacked both Mϕ and T cells, and the B cell population was unresponsive to the B cell mitogen LPS. However, proliferative B cell responses were restored by addition of exogenous T cells and Mϕ or 2-mercaptoethanol (Kagnoff *et al.*, 1974). Others were unable to confirm that T cells are required for LPS mitogenic responses (Gronowicz and Coutinho, 1975). Kagnoff has further suggested that Mϕ are not required for the induction of Th cells in GALT, since mice that were fed erythrocytes exhibited good Th cell function for *in vitro* immune responses (Kagnoff, 1975; Kagnoff and Campbell, 1974; Kagnoff *et al.*, 1974). This accessory cell deficiency was suggested to help explain why final plasma cell differentiation and subsequent local immune responses fail to occur in GALT.

Recent studies have shown that enzymatic treatment of isolated murine PP releases all of the major lymphoreticular cell types, including lymphocytes B and T and accessory cells, i.e., macrophages (Mϕ) and dendritic cells (Frangakis *et al.*, 1982; Kiyono *et al.*, 1982b). Approximately equal numbers of T and B cells are obtained by this method (Fig. 6). Although most of the B cells bear surface IgM (sIgM$^+$) (85–92% of total B cells), a significant percentage of B cells bear surface IgA (sIgA$^+$). It is well known that GALT represents a major IgA inductive site, and higher frequencies of precursors for this isotype are present in PP than in other lymphoid tissues. GALT also contains significant numbers of mature T cells (Fig. 6), most of which bear Lyt-1$^+$ and L3T4$^+$ (a characteristic of inducer–helper T cells), although significant numbers of suppressor–cytotoxic phenotypes (Lyt-2$^+$) are also in evidence. Furthermore, PP possess regulatory con-

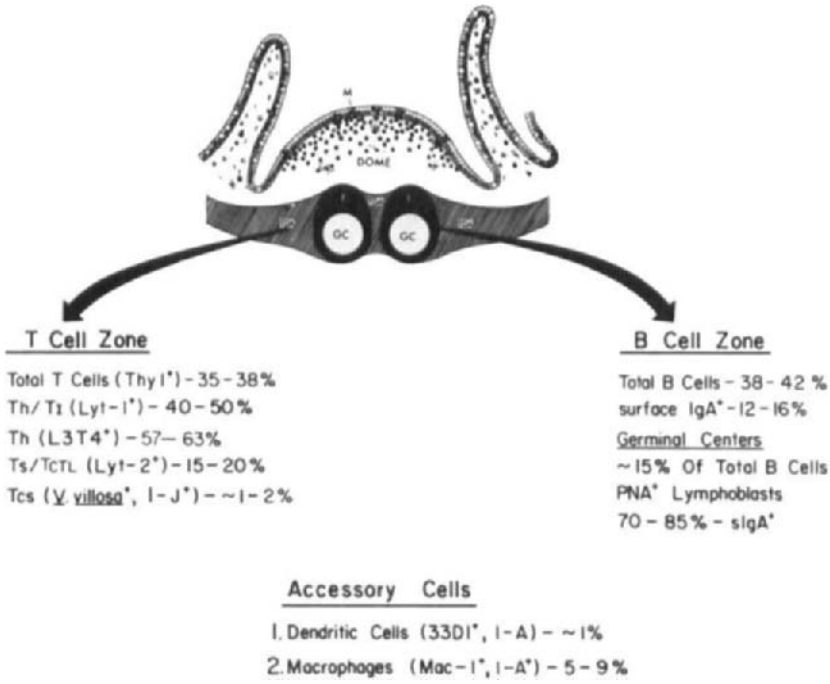

FIG. 6. Morphology of Peyer's patches, and the distribution of T cell and B cell zones with special characteristics of lymphoreticular cells present. Abbreviations: F, follicles; GC, germinal center; M, microfold or follicle-associated epithelial (FAE) cell; TDA, thymus-dependent area.

trasuppressor T cells, which potentiate immune responses to orally encountered antigens (see the section, "Regulation of the IgA Response"). Enzyme dissociation of PP also releases significant numbers of esterase-positive cells (Mϕ) which exhibit phagocytic functions. Finally, I-A$^+$ dendritic cells are present in these cell populations and are fully functional in *in vitro* cultures (Spalding *et al.*, 1983). These dissociated PP cell cultures support full immune responses to both TD and TI antigens. Furthermore, PP cell cultures from mice that are orally primed with TD antigen produce *in vitro* secondary immune responses, especially of the IgA isotype (Kiyono *et al.*, 1982b, 1983; Eldridge *et al.*, 1983).

However, these results with dissociated cells still cannot explain why in GALT there are no local immune responses to antigens encountered via the oral route. It is well known that distinct T and B cell zones are found in GALT (see above), and it is possible that Mϕ are physically separated from areas where T and B cell interactions occur.

It is tempting to speculate that antigen processing by Mϕ in either T cell or B cell zones with subsequent presentation to T or B lymphocytes would result in initial induction of immune responses. The terminal inductive stimuli required for immune responses may occur in distant mucosal tissues after departure of these cells from PP. Additional work remains to be done to determine why T and precursor IgA B cells selectively migrate to distant mucosa and the microenvironmental conditions that determine final differentiation of B cells into fully mature plasma cells that synthesize IgA.

D. Common Mucosal Immune System in Animal Models and in Humans

It has been demonstrated in many experimental models that local antigenic stimulation of mucosal surfaces results in local production of antibodies which appear in mucosal and glandular secretions and also in the circulation (for review see Heremans, 1974). IgA antibodies from the circulation may also contribute to various external secretions, including milk and bile (Heremans, 1974; Virella *et al.*, 1978; Halsey *et al.*, 1980, 1982; Dahlgren *et al.*, 1981; Vaerman *et al.*, 1982; Lemaitre-Coelho *et al.*, 1982; Russell *et al.*, 1982; Sheldrake *et al.*, 1984), depending on the animal species used in such experiments. In humans, however, only minute amounts of IgA from the circulation are transported into external secretions such as saliva and gastrointestinal fluid (Delacroix *et al.*, 1982a; Jonard *et al.*, 1984; Kubagawa *et al.*, 1987).

Heremans and Bazin (1971), postulated that the IgA-producing cells found in the gut of orally immunized germ-free mice are also disseminated into extraintestinal lymphoid organs, including mesenteric lymph nodes and spleen where they produce IgA antibodies specific for ingested antigen (ferritin or sheep erythrocytes). Craig and Cebra (1971) convincingly demonstrated the same year that lymphoid cells taken from rabbit Peyer's patches and appendix and injected into an irradiated allogenic recipient can repopulate the intestinal tissue of such a recipient with IgA-producing cells of the donor type; lymphoid cells from peripheral lymph nodes did not exhibit this potential. Therefore, they proposed that rabbit Peyer's patches are an enriched source of precursors for IgA-producing immunocytes that have the potential to populate mucosal tissues and secretory glands where they differentiate into IgA producers. These studies were extended by Rudzik *et al.* (1975a,b), who also investigated organized bronchus-associated lymphoid tissues (BALT) and demonstrated similarities between BALT and GALT: lymphoid accumulations of the bronchus

were rich in IgA precursors and transfer of such cells into a recipient resulted in the repopulation of lamina propria of both the gut and bronchi with IgA plasma cells of donor origin (Rudzik *et al.*, 1975b).

In addition to respiratory and intestinal tracts, the IgA precursor cells from GALT and BALT also populate remote secretory sites such as mammary, salivary, lacrimal, and uterine cervical glands (Lamm, 1976; Roux *et al.*, 1977; Bienenstock *et al.*, 1978; McDermott and Bienenstock, 1979; Jackson *et al.*, 1981; Montgomery *et al.*, 1983), where they differentiate into IgA plasma cells. In addition to the commitment to produce IgA, the cells from BALT and GALT are sensitized in these tissues by antigens present in the gut or bronchial lumen and produce, after local differentiation into IgA plasma cells, antibodies that are specific for an ingested or inhaled antigen. Montgomery *et al.* (1974) orally immunized lactating rabbits with DNP-labeled pneumococcus and induced DNP-specific IgA antibodies in milk. Because such antibodies are not selectively transported into the milk from the circulation and the fact that the authors elegantly excluded the stimulation of IgA antibody-producing cells in the mammary gland by absorbed circulating antigen (by concomitantly inducing high levels of circulating anti-DNP IgG antibodies) (Montgomery *et al.*, 1976) these authors suggested that antigen-committed IgA precursor cells from GALT seeded the mammary glands. Robertson and Cebra (1976) demonstrated that stimulation with soluble or particulate antigen of a Peyer's patch-containing isolated ileal loop results in an IgA immune response in another isolated intestinal loop. These findings directly implicated the Peyer's patches as the site of precursor cells capable of undergoing antigen-driven stimulation to proliferate, mature, migrate, and repopulate the intestinal lamina propria with cells capable of secreting specific IgA antibodies. Michalek *et al.* (1976) also demonstrated that rats fed *Streptococcus mutans* responded by the production of S-IgA in external secretions in the absence of a significant serum antibody response. Similar experiments using a variety of other microbial antigens have been performed in numerous animal experimental systems (Cebra *et al.*, 1977). Particularly important were studies of Weisz-Carrington *et al.* (1979), who have shown that oral administration of ferritin results in the appearance of specific antibody-producing cells in extraintestinal lymphoid tissues of mice. Furthermore, in secretory tissues the immunoglobulin isotype of anti-ferritin-producing plasma cells was mostly IgA, while such cells in the spleen or peripheral lymph nodes were usually of IgM or IgG isotypes. The most convincing experiment which demonstrated the dissemination of antigen-sensitized IgA precursors into

exocrine tissues utilized the transfer of lymphocytes from mesenteric or peripheral lymph nodes of orally immunized animals. IgA anti-ferritin cells preferably homed to intestines and lactating mammary glands whereas IgM and IgG anti-ferritin cells homed to peripheral lymph nodes. DeBuysscher and Dubois (1978) detected anti-*Escherichia coli* plasma cells in the intestine and salivary glands of pigs orally and locally infected with this bacterium. In rabbits Jackson and Mestecky (1981) detected IgA anti-human serum albumin (HSA) in the gut and parotid gland of animals orally immunized with HSA.

Evidence for a common clonal origin of cells that homed to different secretory tissues was strengthened by immunochemical analyses of IgA in several external secretions. Using isoelectric focusing Montgomery *et al.* (1983) determined the spectrotype of IgA anti-DNP antibodies in tears, saliva, and bronchial fluid of orally immunized rats. Although spectral components of anti-DNP IgA from different animals did not match, the isolectric points for specific IgA from tears, saliva, and bronchial fluid of each individual rat were identical. These findings indicate that the lacrimal and salivary glands as well as bronchial mucosa were populated by cells of a common clonotype.

In summary, enough evidence has been accumulated that experimentally proves the existence of a common mucosal immune system (for details see Cooper *et al.*, 1974; Lamm, 1976; Cebra *et al.*, 1977; Mestecky *et al.*, 1978a, 1980b; Bienenstock, 1978, 1979) (Fig. 7). The major natural pathway for stimulation of the immune system occurs through GALT (and BALT) where environmental or artificially introduced antigens penetrate through highly pinocytotic and phagocytotic M cells covering GALT and BALT and interact with resident accessory and lymphoid cells. B cells that contain a large number of IgA precursors leave GALT and BALT, enter regional lymph nodes (for example mesenteric), and enter the general circulation through the thoracic duct. They then home by mechanisms described below to the lamina propria of intestinal and respiratory tracts, salivary, lacrimal, mammary, and cervical uterine glands where they differentiate into IgA plasma cells producing antibodies specific for ingested or inhaled antigen.

1. Common Mucosal Systems in Humans

The above-described experiments performed in various animal models clearly demonstrated that mucosa-associated lymphoid tissues such as Peyer's patches and BALT are the primary source of antigen-sensitized and isotype-restricted cells that populate remote secretory sites. However, the most convincing evidence is based on experi-

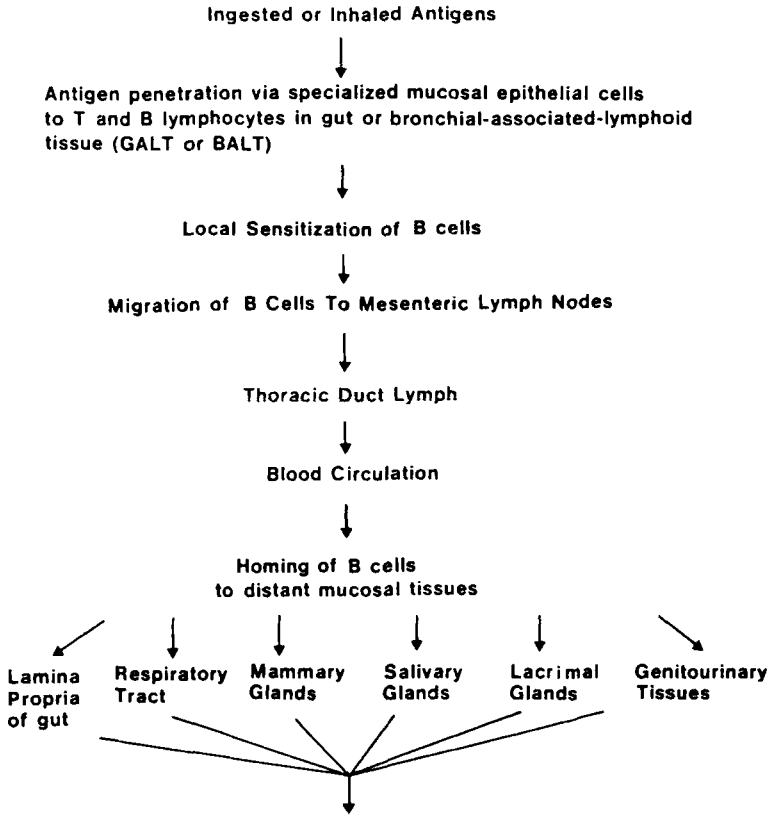

FIG. 7. Common mucosal immune system. Pathway of the migration and homing of antigen-sensitized precursors of IgA-producing plasma cells to various mucosa-associated tissues and glands has been demonstrated in experiments performed in different animal models. In humans, precursors of IgA plasma cells have been detected in the peripheral blood of volunteers orally immunized with microorganisms and their products. For details see Section IV,D.

ments in which labeled cells from various lymphoid tissues of the donor were transferred into irradiated, syngeneic recipients. Because this experimental approach cannot be used in humans, the evidence for the existence of a common mucosal system remains indirect. Nevertheless, despite these obvious limitations, there is an increasing body of information that indeed suggests that an analogous common mucosal immune system is operational in humans.

The first line of evidence is based on the presence of natural or artificially induced S-IgA antibodies to various microbial antigens in

secretions of glands that are not directly stimulated with such antigens. For example, human colostrum and milk contain S-IgA antibodies to an oral bacterium, *Streptococcus mutans* (Arnold *et al.*, 1976). These antibodies are present in colostrum before the onset of nursing and are unlikely to be the result of retrograde stimulation of the mammary gland by bacteria from the oral cavity of the baby. *S. mutans* colonizes tooth surfaces but not mucous membranes. S-IgA antibodies to this microorganism are also present in tears and parotid saliva (Arnold *et al.*, 1976; Allansmith *et al.*, 1983). Because S-IgA in these secretions is not derived from the circulation but is locally produced in the glandular tissue, it follows that the precursors of IgA-producing plasma cells that homed to these glands were committed to produce anti-*S. mutans* antibodies in the lymphoreticular tissues of the intestine. This point has been further addressed in studies concerning the induction of S-IgA antibodies to *S. mutans* by oral immunization (see below).

Although naturally occurring IgA antibodies to a broad spectrum of microbial and food antigens have been detected in human colostrum and milk (Allardyce *et al.*, 1974; Hanson *et al.*, 1983; Ogra *et al.*, 1983; Mestecky *et al.*, 1985), the strict criteria for association of such antibodies with S-IgA (polymeric IgA containing SC) have not always been fulfilled. Nevertheless, several studies summarized by Mestecky *et al.* (1985), in which a variety of sensitive techniques have been used to identify S-IgA, clearly indicate that secretions of glands not directly stimulated with these antigens contain S-IgA antibodies.

Further experimental evidence for the existence of the common mucosal immune system in man has been provided by oral immunization of volunteers with microbial antigens. Goldblum *et al.* (1975) infected the intestinal tract of lactating mothers with live *Escherichia coli* and observed IgA antibodies in colostrum and milk to lipopolysaccharide from the infecting bacterium. Subsequent studies with large doses of killed and lyophilized *S. mutans* given orally in gelatin capsules indicated that S-IgA antibodies to the whole bacterium were induced in parallel in whole, submandibular, sublingual, and parotid saliva as well as in tears (Mestecky *et al.*, 1978b). S-IgA anti-*S. mutans* antibodies increased rapidly and reached the highest levels on days 10–25 and decreased during the two ensuing months. Subsequent oral challenge with the same antigen led to the appearance of S-IgA antibodies that resembled secondary immune responses. Preexisting serum levels of IgA, IgG, or IgM anti-*S. mutans* antibodies were not changed as a consequence of oral immunization.

In another study (Clancy *et al.*, 1983), oral immunization with a killed polyvalent bacterial vaccine (Buccaline Bern, Switzerland) was given to newborns at birth and at 1 and 2 months. Salivary but not serum IgA and IgG responses to *Haemophilus influenzae* (3.5×10^{10} bacteria per each immunization course) was detected using a class-specific solid phase radioimmunoassay.

In extensive studies of oral immunization with a viral antigen Waldman *et al.* (1983, 1986) used, for seven consecutive days, enterically coated gelatin capsules containing commercially available parenteral influenza vaccine. Levels of IgA, IgM, and IgG antibodies to hemagglutinin were examined by a sensitive ELISA technique in serum, nasal wash, and saliva. Levels of specific IgA antibodies were increased in nasal wash and saliva but not in serum.

Because of the unavailability of lymphoid tissues from orally immunized volunteers the cellular aspects of the IgA immune responses were not investigated in any of the studies so far performed. As described above (IgA cell cycle in animal models), antigen-sensitized precursors of IgA plasma cells leave GALT and through the thoracic duct enter the general circulation. Therefore, analyses of cells from the peripheral blood of orally immunized individuals may shed light on the cellular kinetics of an IgA immune response. Previous studies in our laboratory (Kutteh *et al.*, 1980; Moldoveanu *et al.*, 1984) suggested that peripheral blood mononuclear cells stimulated with pokeweed mitogen, Epstein–Barr virus, or lipopolysaccharide secrete predominantly polymeric IgA. Experimental evidence that this was indeed polymeric IgA rather than aggregates was provided by the elution position of IgA released into culture supernatant on a calibrated gel filtration column, the presence of J chain, sensitivity to reduction of disulfide bonds (Kutteh *et al.*, 1980), and the ability to bind SC (Moldoveanu *et al.*, 1984). Furthermore the proportion of IgA_1- and IgA_2-positive cells, as determined by immunofluorescence with monoclonal antibodies (Kutteh *et al.*, 1980; Conley *et al.*, 1980), resembled that of secretory tissues but not of bone marrow, spleen, or peripheral lymph nodes (Crago *et al.*, 1984). Therefore, we concluded that peripheral blood contains a population of lymphocytes with the potential to exhibit, after mitogen stimulation, characteristics similar to IgA cells found in external secretory tissues [predominance of polymeric IgA and proportion of IgA_1 and IgA_2 cells (Mestecky *et al.*, 1982)]. The detection of such cells in the peripheral circulation suggests that these cells are precursors of IgA-producing plasma cells with the potential to populate mucosal tissues. Additional support for this conclusion was provided by earlier studies of the levels of serum

and salivary IgA in patients undergoing extracorporal cesium irradiation of peripheral blood (Friedman *et al.*, 1975). A dramatic decrease of salivary IgA levels was observed, whereas the serum levels remained unchanged. It should be remembered that in humans, serum IgA is predominantly produced by plasma cells in the bone marrow and secretory tissues appear to contribute little to the circulatory pool (see above).

Further evidence for the presence in the peripheral blood of IgA cells that produce antibodies specific for ingested antigen came from recent studies concerning oral immunization with *Vibrio cholerae* vaccine (Lycke *et al.*, 1985) and *S. mutans* (Mestecky *et al.*, 1986). Lycke *et al.* (1985) demonstrated that lymphocytes from the peripheral blood of individuals orally immunized with a single dose of a combined cholera B subunit/whole cell vaccine spontaneously secreted antibodies predominantly of the IgA isotype into culture supernatants. In our study (Mestecky *et al.*, 1986), five of six adult volunteers who ingested enterically coated gelatin capsules that contained killed and lyophilized *S. mutans* responded by the production of S-IgA (or IgM in one IgA deficient female) in saliva and tears but not in serum. Peripheral blood mononuclear cells were found to secrete spontaneously anti-S. *mutans* antibodies as detected by an enzyme-linked immunospot (ELISPOT) assay developed by Czerkinsky *et al.* (1983). Spot-forming cells were detected as early as day 7 after onset of oral immunization and reached peak frequencies by days 10–12. A rapid decrease to preimmunization levels was seen between days 14 and 21. IgA anti-S. *mutans*-secreting cells accounted for most of the spots detected and IgM cells, though less frequent, followed a similar pattern. Examination of mononuclear cells and culture supernatants after polyclonal expansion with pokeweed mitogen revealed that the peripheral blood contains, after oral immunization, a population of antigen-sensitized precursors of IgA cells capable of *in vitro* production of IgA antibodies to the ingested antigen. Parallel examination of external secretions for anti-S. *mutans* activity showed increased levels by day 21. Therefore, the appearance of spot-forming cells in the peripheral blood preceded the appearance of specific antibodies in secretions.

Taken together, the available data strongly suggest that a common mucosal immune system, analogous to that described in various animal models, is also operational in humans. Although the doses and forms of various antigens may substantially influence the magnitude of the S-IgA in external secretions, oral immunization has proved to be the route which should be seriously considered for the prevention of

infectious diseases encountered through or affecting large areas of mucosal surfaces.

2. *Mechanisms of Homing of Precursors of IgA-Producing Plasma Cells to Mucosal Tissues and Secretory Glands*

Properties of IgA precursor cells that home to secretory tissues and glands have been studied in animal models using radioactively labeled cells from various sources. Gowans and Knight (1964) observed that large lymphocytes from the thoracic duct lymph did not recirculate through the lymphatics and blood vessels, but rapidly homed to the intestinal lamina propria. Many subsequent studies corroborated these findings and led to the generally accepted conclusion that the source of lymphocytes governs the site to which they migrate. Donor lymphocytes from mesenteric lymph nodes or thoracic duct but not from peripheral lymph nodes of rats and mice homed within a few hours to lymphoid tissues associated with the gut and secretory glands (Griscelli *et al.*, 1969; Guy-Grand *et al.*, 1974). A higher percentage of large lymphocytes that populate these tissues express surface IgA (Jones and Cebra, 1974) and treatment of these cells with anti-α chain reagent and complement effectively abrogated their homing.

Several possible mechanisms responsible for the preponderance of IgA plasma cells in mucosa-associated tissues and secretory glands have been considered in many investigations that addressed this puzzling phenomenon (for review see Bienenstock, 1982).

Because of the intimate anatomical association and functional dependence (IgA transport) of the IgA-producing plasma cells and epithelial cells in secretory tissues, product(s) of the latter cell type, such as SC, have been suspected to play a decisive role in this process (Craig and Cebra, 1971; Brandtzaeg, 1973). In a subsequent experiment concerning this point Setcavage *et al.* (1976) demonstrated that porcine mononuclear cells indeed expressed a receptor for SC on their surface membrane. Furthermore, in a case of SC deficiency, subepithelial tissues of a patient were devoid of IgA plasma cells (Strober *et al.*, 1976). Contrary to these findings, rat (Husband and Gowans, 1978) or human (Brandtzaeg, 1976d; Crago and Mestecky, 1979) peripheral blood mononuclear cells before and after pokeweed mitogen stimulation as well as IgA lymphoblastoid cell lines failed to bind SC on the surface. However, after fixation, cytoplasmic staining with SC was observed in such cells. In addition, fixed sections of secretory tissues demonstrate affinity for SC (Brandtzaeg 1973, 1983; Moldoveanu *et al.*, 1984), apparently due to the presence of small

amounts of polymeric intracytoplasmic IgA. In contrast, IgA found on the surface of IgA-bearing lymphocytes (Williams and Gowans, 1975; McWilliams *et al.*, 1977) occurs in monomeric form and is unlikely to interact significantly with SC. Furthermore, systemic injection of anti-SC or IgA failed to diminish the homing of IgA precursors in mice (McWilliams *et al.*, 1975). Therefore, it is unlikely that SC serves as a homing receptor for precursors of IgA-producing plasma cells. Other, as yet incompletely characterized, products of mouse mammary gland found in milk whey have been shown to display *in vitro* chemotactic activity for IgA- and also IgG-bearing lymphocytes from mesenteric but not peripheral lymph nodes (Czinn and Lamm, 1986). Surprisingly, surface IgM-positive cells and T lymphocytes were unaffected. This was particularly relevant to earlier reports clearly demonstrating that secretory tissues of IgA-deficient individuals are populated by IgM-producing plasma cells (Brandtzaeg *et al.*, 1968) and this isotype can functionally replace IgA in such patients (Arnold *et al.*, 1977, 1978).

Early studies in germ-free animals have suggested that microbial colonization of the intestinal tract results in the appearance of large numbers of IgA-positive cells in the lamina propria (Crabbe *et al.*, 1968). In addition to stimulating the immune response, locally applied antigens have also been considered as being responsible for attraction of corresponding precursors of IgA immunocytes into secretory tissue (Gowans and Knight, 1964). This notion gained support from experiments reported by Pierce and Gowans (1975) and Tlaskalova *et al.* (1983). In the latter experiments the presence of gut microflora increased the homing of mesenteric lymph node cells into the gut of conventional rats. Comparison of the homing of lymphoid cells obtained from mesenteric lymph nodes of conventional vs germ-free animals suggested that activation of cells by antigens from microflora increases their homing affinity for mucosal surfaces. Using Thiry-Vella intestinal loops, Husband and Gowans (1978) concluded from their experiments that the circulating cells which give rise to antibody producing immunocytes can migrate into the lamina propria independently of antigen. However, locally present antigen had a profound effect on the magnitude and persistence of the response, due to the local antigen-driven proliferation of clones of antibody-forming cells and their precursors. Furthermore, selective homing also occurs to glands such as mammary and salivary glands (Roux *et al.*, 1977; Weisz-Carrington *et al.*, 1979) and ectopic grafts of fetal gut (Ferguson and Parrott, 1972; Halstead and Hall, 1972; Ferguson, 1974) in the absence of antigen.

Another attractive explanation for selective distribution of lymphocytes from various lymphoid organs into the predestined site of homing was provided by experiments from Weissman's and Butcher's groups. Specific interactions between receptors on high endothelial cells of postcapillary venules and lymphocytes were observed (Butcher *et al.*, 1982a). Using both *in vitro* and *in vivo* assays, these authors demonstrated characteristic preferential distribution of B and T cells into various lymphoid organs, including Peyer's patches and mesenteric and peripheral lymph nodes. Whether circulatory precursors of IgA-producing cells emigrate into the lamina propria of the gut and secretory glands by analogous mechanisms has not been demonstrated as yet.

There are alternative mechanisms that may explain the preponderance of IgA plasma cells in secretory tissues. First, B cells may lodge in such tissues irrespective of their potential to produce IgA but the microenvironment in such tissues allows preferential differentation of IgA precursor cells (Pittard and Bill, 1979a,b). Epithelial cells, accessory cells, or isotype-specific immunoregulatory T cells and their products (see Section IV,E) may account for the suppression of differentiation of non-IgA precursors and help for maturation into IgA precursors. Thus, the selective distribution of such immunoregulatory cells may influence local differentiation of arriving B cells. However, secretory tissues of some IgA-deficient individuals are populated by IgM immunocytes (Brandtzaeg *et al.*, 1968) and in some tissues, such as lacrimal glands (Brandtzaeg *et al.*, 1979), a relatively high percentage of total immunoglobulin-containing cells produce IgD. In inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, large numbers of IgG immunocytes have been detected (Baklien and Brandtzaeg, 1975). Therefore, the selectivity of homing of B cells may not be restricted to IgA plasma cell precursors.

A large proportion of cells in human peripheral blood differentiates, after pokeweed mitogen stimulation, into IgA producers. High numbers of IgA precursors in the peripheral blood may be needed for continuous replenishment of IgA plasma cells in secretory tissues which have a rather short life span in their terminal differentation stage. Therefore, the influx from the circulation of large numbers of cells that have the potential to differentiate into IgA producers, together with the local glandular microenvironment, may account for the preponderance of IgA plasma cells in such tissues. It is usually overlooked that after pokeweed mitogen stimulation of human peripheral blood mononuclear cells IgA-positive cells often predominate (Mestecky *et al.*, 1977; Al-Balaghi *et al.*, 1984), and that these cells secrete predominantly polymeric IgA and display almost identical

proportions of IgA_1- and IgA_2-positive cells (Kutteh *et al.*, 1980). These findings strongly suggest that the peripheral blood is indeed rich in precursors that are destined to populate mucosa-associated tissues and secretory glands.

E. Factors Regulating S-IgA Immune Responses

It is now clear that the mode of induction and regulation of IgA expression is separate from the systemic (blood-borne) immune system (McGhee and Mestecky, 1983; Strober *et al.*, 1982). Peyer's patches are unique with respect to their function as IgA-inductive sites as well as being present in a position of constant exposure to gut biostimulants including the outer membrane components of gram-negative bacteria. As discussed elsewhere, all major lymphoreticular cells are present in GALT for immune response induction, including precursors of IgA B cells, regulatory T cells, and functional accessory cells. However, antigenic stimulation does not result in local responses, and this is due in part to regulatory T cell influences. In addition, antigen can induce negative regulatory responses in PP, e.g., T suppressor cells which migrate to peripheral lymphoid tissue and mediate systemic unresponsiveness (see the section on oral tolerance). Thus, in studying the regulation of responses in GALT one must consider environmental biostimulants such as endotoxin, as well as specific responses regulated by T cell subsets and unique accessory cells for induction and regulation of IgA responses and oral tolerance.

1. Environmental Antigen Influences on GALT

Natural antigen priming and bacterial mitogen stimulation exert strong influences on lymphoreticular cell development in GALT. This affects both the B cell compartment (including commitment to IgA) as well as precursors of regulatory T cells. Using clonal dilution analysis Shahin and Cebra (1981) have shown that natural antigens (haptens), e.g., inulin, phosphocholine (PC), or Dextran (α1–6 Dex), increase the frequency of responsive B cell clones. For example, B cells from neonatal mice possess low numbers of clonal precursor B cells to these antigens (Shahin and Cebra, 1981; Sigal *et al.*, 1977; Fernandez and Möller, 1978), however, a sharp rise occurs with anti-phosphocholine (PC) precursors between days 9 and 14 of neonatal life (Sigal *et al.*, 1977). Similar increases in anti-inulin precursors occur at 3–5 weeks of age, while anti-α1–6 Dextran precursors reach significant levels at 3 months of life (Shahin and Cebra, 1981; Fernandez and Möller, 1978). The majority of these B cell precursors support T cell-dependent (TD)

clones that secrete IgM, together with IgG and/or IgA (Shahin and Cebra, 1981; Cebra *et al.*, 1983; Gearhart and Cebra, 1979). One possible explanation for these increases would be a natural priming by environmental antigens which induce competent B cells to clonal isotype switching (Cebra *et al.*, 1984). Strong support for this concept came from clonal analysis of PP B cells (Gearhart and Cebra, 1979; Cebra *et al.*, 1976). It is well established that PP contain higher frequencies of B cells already committed to differentiation into IgA plasma cells than any other lymphoid tissue (Craig and Cebra, 1971, 1975). Clonal analysis of PP B cells showed that 40–60% of clones with specificity for anti-PC, anti-inulin, and anti-β galactose exclusively expressed IgA, while B cells of virgin antigen specificities, e.g., anti-DNP, yielded mixtures of IgM, IgA, and IgG. These latter clones were similar to those obtained from spleens of the same mice (Gearhart and Cebra, 1979; Cebra *et al.*, 1976, 1983). This analysis strongly suggests that a selective priming of PP B cells occurs by naturally encountered antigens and results in subsequent restriction in the isotype potential of B cells to IgA expression (Cebra *et al.*, 1984).

Continuous exposure to environmental antigens present in the gut lumen exerts regulatory effects on immunocompetent cells. A gram-negative bacterial flora and released cell wall components such as endotoxin or lipopolysaccharide (LPS) significantly affect lymphoreticular cells in the PP (McGhee *et al.*, 1984). Dubos and Schaedler (1960) suggested that the endogenous gut microflora has a marked effect on the hosts' susceptibility to infection. Evidence has been presented that germ-free mice are susceptible to infection (Dubos and Schaedler, 1960) but resistant to the lethal effects of LPS (Jensen *et al.*, 1963; Schaedler and Dubos, 1961, 1962). On the other hand, mice which possess a gram-negative gut microflora are more susceptible to the lethal effects of LPS (Jensen *et al.*, 1963; Schaedler and Dubos, 1962; Kiyono *et al.*, 1980b). These results indicate that endogenous gut LPS influences the hosts' susceptibility to gram-negative infection, perhaps via an interaction with PP lymphoreticular cells and the subsequent induction of innate host immunity to infection.

The C3H/HeJ mouse strain has proved useful for study of lymphoreticular cell interactions involved in the induction of IgA responses in the absence of LPS effects which occur in normal hosts. This is due to the inability of the C3H/HeJ mouse to respond to LPS, or more precisely to the biologically active lipid A component, a trait regulated by the Lps^n gene (n = normal) in normal or responsive mice. The *Lps* gene is defective (d) in the LPS nonresponsive C3H/HeJ

strain (Lps^d/Lps^d) (Morrison and Ryan, 1979). Oral administration of TD antigens, such as sheep red blood cells (SRBC) or whole bacteria to C3H/HeJ mice, induce higher IgA splenic plaque-forming cell (PFC) and secretory antibody responses than are seen in identically treated, syngeneic, LPS-responsive C3H/HeN mice (Kiyono *et al.*, 1980a, 1982c). Oral immunization induces greater Th cell activity in PP and spleen of C3H/HeJ mice than that noted in LPS responsive mice, and these Th cells are mainly responsible for the elevated IgA response pattern seen (Kiyono *et al.*, 1980a). Therefore, this mouse strain has been used to study the induction of Th cells in GALT and their precise function in regulation of IgA-specific immune responses (Kiyono *et al.*, 1980a, 1982c,d, 1984a).

2. *T Cell Regulation of the IgA Response*

Although it is now well established that T lymphocytes regulate the immune response, the precise cell interactions and soluble mediators involved are only partially understood. T cells recognize foreign antigen in association with self-histocompatibility (MHC) determinants (Schwartz, 1985), and significant progress has been made in defining the gene organization and structure of the T cell receptor (TCR) (Acuto *et al.*, 1985; Davis, 1985). Tα and Tβ genes code for the disulfide-linked α and β chains which form the clonotypic TCR (Acuto *et al.*, 1985; Davis, 1985), and striking similarities exist between the TCR (Collins *et al.*, 1985; Yanagi *et al.*, 1984; Hedrick *et al.*, 1984; Siu *et al.*, 1984; Gascoigne *et al.*, 1984; Kavaler *et al.*, 1984) and an antibody molecule, where diversity is produced by VDJ and constant region gene recombination. The TCR provides clonal diversity for T cell responses to antigens in nature in a manner analogous to B cell membrane immunoglobulin (Ig) of a single specificity where antigen binding initiates clonal antibody responses.

In the case of T cells, antigen must be seen in association with identical MHC, and antigen-MHC binding to the TCR leads to T cell activation and the release of lymphokines involved in B cell activation (Howard and Paul, 1983; Kishimoto, 1985). These lymphokines interact with receptors which induce B cell growth, proliferation, and differentiation into plasma cells which synthesize antibodies. Current dogma suggests that T cell-derived lymphokines for B cell development first interact with a resting, mature B cell (surface IgM^+, $sIgD^+$). B cell growth and proliferation are induced by antigen and T cell-derived B cell stimulating factors (BSF), formerly termed B cell growth factor (BCGF) I and II (Howard and Paul, 1983; Kishimoto, 1985). BSF induces B cell proliferative responses, but requires an

initial growth stimulus, e.g., antigen or anti-μ or the putative B cell activation factor (BCAF). BSF expands antigen-specific B cell clones and presumably induces receptors for T cell-derived differentiation factors (BCDF or TRF) which allow the development of B cells into plasma cells synthesizing antibodies (Kishimoto, 1985). To date most workers with BSF and BCDF/TRF have used *in vitro* assays for IgM synthesis, and have thus avoided the important question of factor requirements for other isotypes, i.e., IgG, IgE, or IgA responses.

The IgA response is considered to be T cell dependent, since athymic nude mice exhibit depressed serum IgA levels (Crewther and Warner, 1972; Luzzati and Jacobson, 1972; Pritchard *et al.*, 1973) and neonatally thymectomized rabbits do not undergo IgA responses (Clough *et al.*, 1971). Furthermore, human T cell dysfunctions, including T suppressor cells for IgA, contribute to this isotype-specific immunodeficiency in some individuals (McFarlin *et al.*, 1972; Waldmann *et al.*, 1976; Atwater and Tomasi, 1978; Levitt and Cooper, 1981; Schwartz, 1980). However, others have shown that analysis of T3, T4, and T8 subsets in IgA deficiency exhibit a normal distribution (Reinherz *et al.*, 1981). The first evidence that PP T cells are involved in IgA responses was provided by Elson *et al.* (1979), who showed that murine PP T cells stimulated with Con A suppressed IgM and IgG but helped IgA isotype expression in LPS-driven B cell cultures. On the other hand, Con A-treated splenic T cells suppressed all three isotypes (Elson *et al.*, 1979).

The role of T cells for antibody responses to carbohydrate antigens, e.g., hapten–Ficoll, pneumococcal polysaccharide, or Dextran, is currently under intensive study (Howard and Paul, 1983; Kishimoto, 1985). These antigens were long considered to be T cell independent (TI), and to induce only short-lived IgM responses. However, more recent studies with these so-called type 2 antigens have shown that T cell-derived growth (BSF_1) and differentiation factors are required for B cell responses (Howard and Paul, 1983; Kishimoto, 1985). In this regard, Trefts *et al.* (1981) have shown that IgA anti-Dextran responses are both T cell and age dependent. IgA responses were induced in T cell-enriched cultures from older mice and the antibodies were α1–3 specific with cross-reactive idiotype shared with IgM (MOPC 104E) and IgA (J558) (Kagnoff, 1979). This work suggests that natural antigen priming (see above) results in IgA anti-α1–3 precursors which required T cell factors for IgA synthesis (Kagnoff *et al.*, 1983). Immune responses to carbohydrates are tightly regulated, and T suppressor (Ts) cells inhibit responses and contrasuppressor T cells (Tcs) enhance this antibody response (see below) (Braley-Mullen, 1984, 1986).

Oral administration of T cell-dependent antigens such as sheep red blood cells (SRBC), whole bacteria, or soluble protein antigen results in Th cell induction in PP (Kiyono *et al.*, 1980b, 1982d, 1983; Richman *et al.*, 1981) and in subsequent IgA responses in spleen and in external secretions (Kiyono *et al.*, 1980b, 1982d, 1983). Furthermore, purified Th cells from PP of mice given antigen by the oral route, when added to normal splenic B cell cultures and immunized with the homologous oral-priming antigen, support IgM, IgG, and mainly IgA responses (Kiyono *et al.*, 1983). The elevated IgA response pattern noted with PP Th cells suggested that isotype-specific Th cells occur in GALT and that such cells may account for the predominance of IgA responses following oral immunization.

Recent studies with cloned T cells from murine PP give support for two separate modes of T cell regulation for the IgA response (Fig. 8). The first mode can be outlined as follows: (1) T cells may induce B cells to undergo isotype switching (e.g., Tsw cells), since cloned Tsw cells have been reported to promote $sIgM^+$ B cells to switch to expression of sIgA (Kawanishi *et al.*, 1983a). These autoreactive Tsw cells did not induce IgA synthesis, however, the postswitched $sIgA^+$ B cells could be induced to differentiate and secrete IgA in the presence of conventional B cell growth and differentiation factors which are derived from activated T cells (Kawanishi *et al.*, 1983b, 1985). (2) Another mode for T cell regulation of IgA responses has been provided by studies of clones of PP T cells bearing Fc receptors for IgA ($Fc\alpha R^+$) (Kiyono *et al.*, 1982a) which selectively promote differentiation of $sIgA^+$ B cells into IgA-producing plasma cells (Kiyono *et al.*, 1984a,b). The experiments which showed this were done in the following way. Since oral administration of TD antigens induces Th cells for IgA responses in PP (Kiyono *et al.*, 1980a, 1982c, 1983), Th cells were isolated from PP of mice intubated previously with SRBC, and the Th cells were cloned and maintained in continuous culture (Kiyono *et al.*, 1982a). The cloned T cells ($Fc\alpha R^+$, Thy-1.2^+, Lyt-$1^+,2^-$), when added to purified B cell cultures, exhibited SRBC-specific helper activity mainly for IgA isotype responses. Hence, the clones were designated PP Th A cells. To determine the nature of the B cells which receive help from the PP Th A cells, PP B cells were separated into $sIgA^+$ and $sIgA^-$ populations by flow cytometry (FACS) or by panning, before culture with PP Th A cells and SRBC (Kiyono *et al.*, 1984a). Cultures depleted of $sIgA^+$ B cells gave diminished IgA responses, while those enriched with $sIgA^+$ B cells gave higher IgA responses than were seen in unseparated B cell cultures (Kiyono *et al.*, 1984a). These results indicate that Thα cells can preferentially collaborate with $sIgA^+$ B cells (Fig. 8). It also seemed significant that

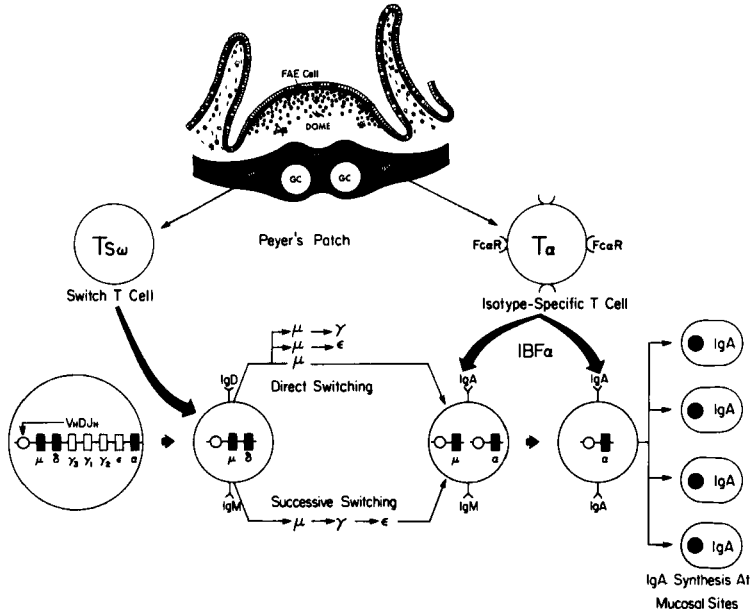

FIG. 8. Two possible mechanisms involved in the regulation of IgA immune responses by two populations of T cells. T switch cells (Tsw) promote, by an unknown mechanism, a switch from the expression of IgM to IgA on surfaces of B cells. The second isotype-specific T cell (Tα) promotes the terminal differentiation of B cells that express IgA into IgA-producing plasma cells at mucosal sites.

the Fcα receptors (FcαR) on cloned Thα cells are important in $sIgA^+$ B cell collaboration for IgA responses (Kiyono *et al.*, 1984a,b).

In contrast to isotype-specific switch T cells, recent studies have indicated the existence of non-isotype-directed switch T cells derived from a patient with the Sezary's syndrome (T_{RAC} cells; Mayer *et al.*, 1982, 1986). This malignant T cell clone induced IgG and IgA switches in normal allogeneic B cell cultures. Furthermore, when B cells were derived from a patient with hyperimmunoglobulinemia M and cultured with T_{RAC} cells, IgG and IgA synthesis occurred (Mayer *et al.*, 1986). However, the levels of IgA produced were quite small and one may question whether this was indeed significant for this isotype (Mayer *et al.*, 1986).

3. *Role of T Cell Fc Receptors for Isotype Responses*

Fc receptors (FcR) are membrane glycoproteins which bind Ig molecules via their Fc region, and have been identified on (1) macro-

phages, (2) B and T lymphocytes, (3) natural killer cells, as well as on (4) nonlymphoid cell types (Möller, 1981; Word *et al.*, 1986). FcR on T cells is important for immunoregulation and several studies have addressed their role in isotype-specific immune responses (Daeron and Fridman, 1985). Initial studies in this area by Fridman *et al.* (1974) showed that alloactivated T cells and derived T cell lines express elevated levels of FcγR (Fridman and Goldstein, 1974), which are released as IgG-binding factors (IBFγ) (Fridman *et al.*, 1974) that can subsequently suppress this isotype *in vitro* (Fridman and Goldstein, 1974; Fridman *et al.*, 1974; Fridman and Gisler, 1975; Neauport-Sautes *et al.*, 1979). Ishizaka and colleagues have extensively studied the induction and function of FcεR$^+$ T cells and IgE-binding factors (IBFε) involved in regulation of the IgE response (Ishizaka, 1983, 1984). A potentiating IBFε binds IgE, is 13,000–15,000 M_r, and is glycosylated with terminal mannose groups which allow its binding by lentil lectin and concanavalin A (Con A) (Suemura *et al.*, 1980; Uede *et al.*, 1982; Uede and Ishizaka, 1982; Yodoi *et al.*, 1980, 1981). A suppressor IBFε has a similar molecular weight, and also binds IgE, but lacks terminal mannose, and is bound by peanut agglutinin (Uede *et al.*, 1982; Uede and Ishizaka, 1982; Yodoi *et al.*, 1981). These two forms of IBFε exhibit a common protein core, and the degree of glycosylation is regulated by glycosylation-enhancing factor (GEF) or -inhibiting factor (GIF) which regulate the production of enhancing or suppressive IBFε, respectively (Uede *et al.*, 1983; Iwata *et al.*, 1983). The gene which codes for the rodent IBFε protein has now been cloned (Martens *et al.*, 1985).

T cells bearing FcαR have been described in both mice and humans (Lum *et al.*, 1979; Strober *et al.*, 1978). Extensive studies by Lynch and co-workers with MOPC 315 IgA myeloma provided the first evidence of IgA-specific immunoregulation (Hoover *et al.*, 1981; Hoover and Lynch, 1981, 1983). It was shown that mice bearing MOPC 315 tumors possess enhanced numbers of FcαR$^+$ T cells, an effect which was due to the high levels of serum monoclonal IgA (Hoover and Lynch, 1981). These FcαR$^+$ T cells were Lyt-1$^-$,2$^+$ and possessed suppressor activity for IgA responses when adoptively transferred into mice orally primed with antigen (Hoover and Lynch, 1983). Additional support for FcαR$^+$ T cell suppression was provided by studies with the FcγR$^+$ and FcαR$^+$ T2D4 cell line (Yodoi *et al.*, 1983). When these cells were incubated with IgA, the FcαR was released as an IgA-binding factor (IBFα) which suppressed IgA synthesis in pokeweed mitogen (PWM)-driven spleen cell cultures (Yodoi *et al.*, 1983). Furthermore, Con A-activated FcαR$^+$ T cells, upon incubation with IgA,

released IBFα and suppressed IgA responses (Adachi *et al.*, 1984). In humans, it has been suggested that FcαR$^+$ T cells help IgA responses, since FACS-enriched FcαR$^+$ T cells from peripheral blood, which consisted of a high proportion of OKT4$^+$ Th cells, selectively enhanced IgA synthesis in PWM-driven B cell cultures (Endoh *et al.*, 1981; Suga *et al.*, 1985).

To examine the molecular basis for the FcαR$^+$ T and sIgA$^+$ B cell collaboration, T–T hybridomas were generated by fusion of the PP Th A cells with a T lymphoma cell line (Kiyono *et al.*, 1985). Sixteen of these T–T hybridomas expressed FcαR, but were FcμR$^-$ and FcγR$^-$. When supernatants from the FcαR$^+$ T cell lines were tested for promotion of IgA responses, all 16 supported antigen-dependent IgA responses in cultures of normal PP B cells (Kiyono *et al.*, 1985). However, supernatants from the 69 FcαR$^-$ cell lines failed to support IgA responses. Passage of the active supernatants over IgA immunoabsorbents removed biological activity, suggesting that the preferential IgA responses are due to IgA-binding factor (IBFα). Active IBFα was eluted from IgA columns, and preincubation of these eluted fractions with purified IgA (but not with IgM or IgG) abrogated their ability to promote IgA responses. To determine whether IBFα can regulate T-dependent IgA responses, purified IBFα was added to SRBC-immunized cultures of unfractionated PP cells from SRBC orally primed mice, a model system previously shown to yield vigorous IgA responses (Kiyono *et al.*, 1982b). High concentrations of IBFα were suppressive, while less IBFα enhanced *in vitro* anti-SRBC PFC responses. Similar results were obtained when splenic B cells were cultured with purified Th cells from PP; higher concentrations of IBFα suppressed, while lower levels enhanced, IgA responses (Kiyono *et al.*, 1985).

These results suggest that Fcα receptors are involved in the T cell selection of sIgA$^+$ B cells and their induction to IgA synthesis by FcαR$^+$ T cells, and they suggest further that relatively high concentrations of IBFα may have a paradoxical suppressive effect. In other systems, it has been shown that released FcαR or IBFα can only suppress polyclonal IgA responses (Yodoi *et al.*, 1983; Adachi *et al.*, 1984). Two alternative possibilities could account for these seemingly conflicting results. A single class of IBFα may be produced by T cells, high levels of which may result in net suppression of IgA responses, whereas moderate levels would enhance Th cell-mediated sIgA$^+$ B cell differentiation with IgA synthesis. Lymphocyte mitogens may exhibit such concentration-dependent paradoxical effects. Alternatively, the IBFα may appear in several molecular forms, analogous to

the immunoregulatory IgE IBFε, where one IBFε class suppresses and a second IBFε enhances the IgE response, the difference apparently depending upon the degree of glycosylation (Ishizaka, 1983, 1984). In this case, our FcαR$^+$ T cell lines may produce a small amount of IBFα of the suppressive form and a greater level of helper IBFα.

A key question in the IgA response involves the relationship between membrane FcαR and secreted IBFα. IBFα may represent the shed FcαR in the culture milieu, or alternatively FcαR and IBFα may be separate molecules, both of which exhibit strong affinity for the Fc of IgA. A second important question is how does the FcαR$^+$ Th cell (and IBFα) collaborate with appropriate B cells? Direct Thα–B cell contact may be mediated by T cell membrane FcαR and the Fc of IgA on the B cell. Secreted IBFα may act as a more conventional B cell stimulating factor with specificity for the IgA-committed B cell subset (Fig. 9). This still would not explain how an antigen-specific Th cell could interact with specific clones of IgA-committed B cells.

One may offer a model to explain how this interaction could occur. PP Th cells may possess the FcαR and the T cell antigen receptor in an associated membrane pattern. If the TCR and FcαR are membrane associated, antigen bridging could occur from antigen presentation by the IgA-committed B cell. The antigen would react with the

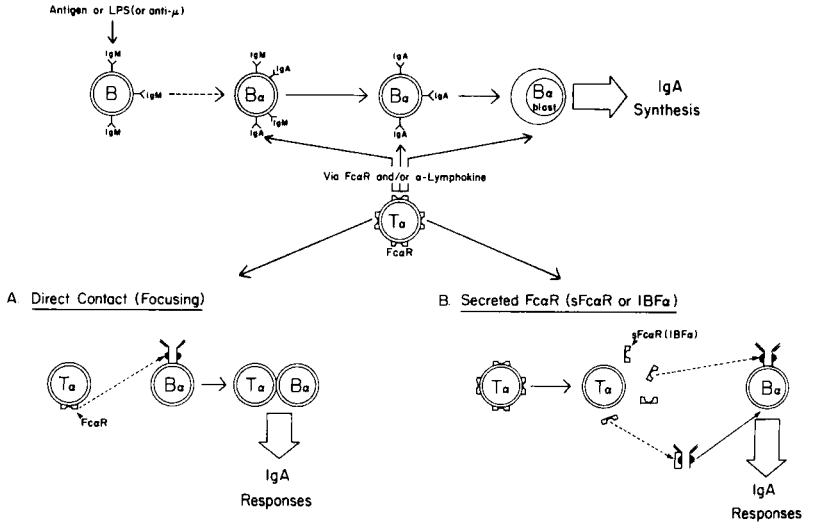

FIG. 9. Possible mechanisms involved in the regulation of IgA synthesis and immune response by Fcα receptor-positive T cells and their soluble product(s)-IBFα.

TCR and the $F(ab')_2$ of membrane IgA, while the FcαR would interact with the Fc region. This may provide a sufficient number of B cell signals required for subsequent development into IgA-producing plasma cells. Tcsα cells also occur in GALT (see below) and may function to protect the FcαR$^+$ Th cell and promote maximum IgA help. Tcsα–Thα cell interactions may occur via either FcαR–anti-FcαR or by FcαR and IgA Fc-like molecules (Fig. 10).

In summary, these results indicate that clones of T cells may produce both cell surface Fcα receptors and a secretory form(s) of IBFα which can profoundly modulate the responses of IgA$^+$ B cells in both upward and down directions. The regulation of IgA antibody responses by IgA-binding factors thus appears to display the same degree of complexity as that seen in the IgE response. An intricate network of IgA-specific immunoregulation is required, since higher vertebrates possess distinct sites predisposed to IgA induction where environmental antigens are encountered, and selective homing mechanisms for redistribution of IgA-committed lymphocytes to distant mucosa where IgA synthesis occurs.

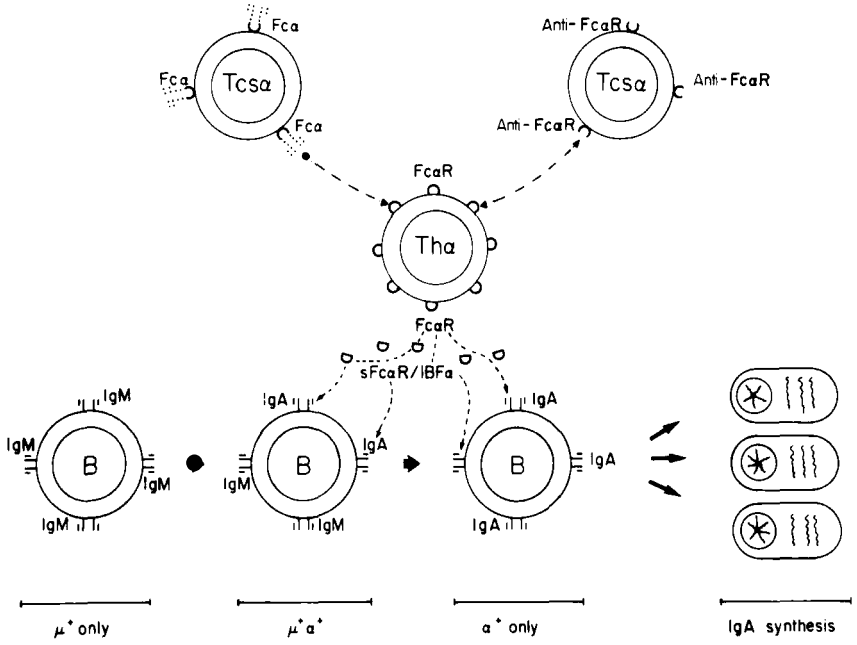

FIG. 10. Model for T cell networks involved in the regulation of IgA synthesis in the mucosal immune system Tα-contrasuppressor cells (Tcsα) may interact with T helper cells (Thα) to support subsequent IgA synthesis.

4. T Cell Networks in the Secretory Immune System

Ingestion of antigen induces two separate immunologic responses, i.e., secretory IgA antibodies at mucosal surfaces and systemic unresponsiveness in peripheral lymphoid tissue such as spleen (Challacombe and Tomasi, 1980). Appropriate doses and spacing of antigen given by the oral route induces Th cells in PP and primes B cells for subsequent IgA responses (Cebra *et al.*, 1984; Kiyono *et al.*, 1980b, 1983; Richman *et al.*, 1981). On the other hand, oral administration of large or prolonged doses of TD antigen results in the inability to respond to this antigen when administered by the systemic route, a condition commonly termed oral tolerance (Tomasi, 1980) (see Oral Tolerance, below). Although oral tolerance may be mediated by immune complexes, by anti-idiotypes, or by other means, most evidence suggests that Ts cells are induced in PP and seed to peripheral lymphoid tissue, such as spleen, where they mediate systemic unresponsiveness (Richman *et al.*, 1981; Kiyono *et al.*, 1982c; Mattingly *et al.*, 1980). Oral tolerance and IgA responses are clearly separate events, since it has been shown that oral administration of protein antigen or bacteria induces simultaneous systemic unresponsiveness and salivary IgA antibody responses (Challacombe and Tomasi, 1980). Thus, antigen uptake into GALT results in the simultaneous induction of Ts cells which migrate to the periphery and mediate oral tolerance and IgA-specific Th cells which, in turn, direct IgA-specific B cell responses for subsequent S-IgA synthesis at distant mucosal sites.

Dual maintenance of IgA-specific Th cells and Ts cells clearly requires another level of regulation, which appears to be provided by the contrasuppressor T cell (Tcs) system. Three cell types are involved in the contrasuppressor circuit, i.e., Lyt-1$^-$,2$^+$, I-J$^+$ Tcs inducers, Lyt-1$^+$,2$^+$, I-J$^+$ transducer cells, and effector Tcs cells which are Lyt-1$^+$,2$^-$, I-J$^+$ (Green *et al.*, 1981, 1983; Gershon *et al.*, 1981; Iverson *et al.*, 1984). Tcs cells prevent Ts cell-mediated suppression of Th cells and convert tolerigenic to immunogenic signals in contact sensitivity reactions (Green *et al.*, 1983; Gershon *et al.*, 1981; Iverson *et al.*, 1984; Ptak *et al.*, 1981). Thus, Tcs cells enhance Th cell-mediated functions. Murine PP are enriched in T cell subsets with helper–amplifier and contrasuppressor activity (Green *et al.*, 1982). Recent studies have shown that Tcs cells can abrogate oral tolerance when adoptively transferred to mice which had been given antigen by the oral route for prolonged periods (Suzuki *et al.*, 1986b). This clearly implies first that oral tolerance is mediated by

Ts cells and, second, that Tcs cells can tip the balance from suppression to antibody responses, presumably by enhancement of Th cell activity.

Evidence for the existence of an isotype-specific Tcs cell subset was provided by the finding that PP Tcs cells bind to immobilized FcαR$^+$ Th cells and adoptive transfer of this subset to tolerant mice potentiated largely IgA responses (Suzuki *et al.*, 1986a). Transfer of nonadherent Tcs cells also abrogated oral tolerance, but only supported IgM and IgG responses (Suzuki *et al.*, 1986a). This suggests that effector Tcs cells in PP directly interact with IgA-specific Th cells for subsequent IgA responses. It may now be useful to consider GALT as a unique immunologic organ when compared with systemic lymphoid tissue such as spleen. In this regard, GALT contains two lymphoid cell compartments. The first (I) would consist of normal B cells (μ^+, δ^+) and regulatory T cells, e.g., Th, Ts, and Tcs cells, which either enhance (or suppress) IgM and IgG subclass responses. Compartment I cells, of course, occur in other lymphoid tissues, and could represent the major cell types present in spleen. The second (II) compartment is unique to GALT and consists of a full repertoire of committed, sIgA$^+$ B cells, IgA-specific Th cells, and a unique population of isotype-specific Tcs cells which act on FcαR$^+$ Th cells for potentiation of IgA responses. Oral administration of TD antigen would perturb both compartments and compartment I responses would favor immunosuppression. Lymphocyte redistribution would ensure that Ts cells are present in peripheral lymphoid tissue for down regulation of responses to antigens which escape from the gut tissues. Compartment II responses involve only the IgA isotype and result in stimulation of IgA B cells and in the induction of FcαR$^+$ Th cells. The latter response would be under the full protection of IgA-specific Tcs cells present in GALT. It should be noted that selective homing of all compartment II cells may occur to mucosal tissues, such as lamina propria regions of the gut. However, it would only be necessary to postulate that committed IgA B cells undergo this process, a fact that is now well established (McDermott and Bienenstock, 1979; Weisz-Carrington *et al.*, 1979). Much remains to be learned about these two major pathways of host responses in GALT, and the precise regulatory processes which control each compartment. However the availability of current molecular and cell biology systems should allow testing of this concept and ultimately address the mechanisms involved in immunological homeostasis to antigens encountered from the gut environment.

5. *Accessory Cells and Isotype-Specific Responses*

Recent studies with enzyme-dissociated murine PP cells has shown that PP contain functional dendritic cells (DC) (Spalding *et al.*, 1983). Studies with PP cell suspensions indicated that DC were present in numbers comparable to those found in spleen. These DC share surface characteristics, including the presence of surface Ia and a DC-specific determinant recognized by a monoclonal antibody 33D1, a lack of surface Ig, T cell markers, or Fc receptors for Ig (Steinman and Cohn, 1973; Steinman *et al.*, 1978; Nussenzweig *et al.*, 1982).

The induction of polyclonal immunoglobulin synthesis has provided a useful method to study B cell activation and differentiation (Coutinho *et al.*, 1974; Coutinho and Möller, 1974; Melchers *et al.*, 1980), as well as the role of accessory cells and of T cells in regulating these events (Kuritani and Cooper, 1982). In accordance with the findings of numerous other investigators (Elson *et al.*, 1979; Kawanishi *et al.*, 1983a; Kearney and Lawton, 1975), we have failed to demonstrate the induction of polyclonal IgA synthesis with various T and B cell mitogens. The addition of 2-mercaptoethanol (2-ME) to PP B cell cultures enhanced IgA synthesis by 5- to 8-fold, leading to the possible involvement of accessory cells in the support of polyclonal IgA responses. Studies with accessory cells in PP have shown that sodium periodate treatment of PP cell cultures results in the formation of large cell clusters composed of DC and T cells. When cells from these clusters were isolated and added to purified PP or splenic B cells, significant polyclonal IgA synthesis occurred (Spalding *et al.*, 1984).

Other studies have suggested that DC–T cell clusters from murine PP induced nontransformed pre-B cells to secrete large amounts of IgA, and more intermediate levels of IgM and IgG. On the other hand, spleen-derived and DC–T cell clusters only induced secretion of IgM (Spalding and Griffin, 1986). These studies imply that GALT DC may act to induce pre-B cells to mature into functional cells and to undergo switches to the expression of IgA (Spalding and Griffin, 1986). The concept of isotype-specific accessory cells is provocative, and much additional work will be required to substantiate this suggestion.

6. *The Immunoglobulin A Heavy Chain Class Switch*

The κ, λ, and heavy (H) chain genes are located on mouse chromosomes 6, 16, and 12, respectively (D'Eustachio *et al.*, 1980, 1981; Swan *et al.*, 1979) and on human chromosomes 2, 22, and 14, respectively (Erikson *et al.*, 1981; Malcolm *et al.*, 1982; McBridge *et al.*,

1982). Three separate gene segments encode the H chain variable (V) region, i.e., V_H (AA residues 1–101), D_H (residues 102–106), and J_H (107–121), where D_H is the diversity segment, J_H the joining segment which is ultimately attached to the constant (C_H) region of the H chain (Honjo, 1983; Early *et al.*, 1980; Kurosawa and Tonegawa, 1982; Kurosawa *et al.*, 1981). The κ or λ chains are encoded by V_L (residues 1–97), J_L (98–110), and C_L (111–218) genes (Honjo, 1983; Bernard *et al.*, 1978; Tonegawa *et al.*, 1978; Max *et al.*, 1979; Sakano *et al.*, 1979). In the pre-B cell, isotype commitment results from rearrangement and joining of D_H and J_H genes and then a V_H gene into a single VDJ complex which is transcribed together with $C_{H\mu}$ to form a complete μ-chain message and subsequently results in cytoplasmic expression (Cooper, 1981; Burrows and Cooper, 1984). This is followed by V_L to J_L rearrangements leading to the expression of a functional VJ–C_κ or VJ–Cλ gene complex and subsequent membrane expression of complete IgM molecules on the newly developed B cell (Cooper, 1981; Burrows and Cooper, 1984; Abney *et al.*, 1978). Diversity results from imprecision in the joining processes, e.g., D_H to J_H and V_H to D_HJ_H and in V_L to J_L recombination (Max *et al.*, 1979; Sakano *et al.*, 1979; Alt and Baltimore, 1982). Due to this junctional diversity, different amino acids are encoded in these regions and the subsequent changes in the hypervariability segments of the VDJ portion of the Ig molecule result in different antibody specificities (Honjo, 1983; Max *et al.*, 1979; Sakano *et al.*, 1979; Alt and Baltimore, 1982). Somatic mutation in the V gene can also increase the diversity and affinity of antibodies (Tonegawa, 1983; Baltimore, 1981; Gearhart, 1982; Rabbitts, 1981).

A major unresolved question about the mucosal immune system is a molecular explanation for the high frequency of IgA-committed B cells which occur at IgA inductive sites, e.g., the PP (Craig and Cebra, 1971; Jones *et al.*, 1974; Rudzik *et al.*, 1975a; Cebra *et al.*, 1980, 1983a). In the mouse, the order of the C_H genes on chromosome 12 is 5′-C_μ-C_δ-$C_{\gamma 3}$-$C_{\gamma 1}$-$C_{\gamma 2}$-C_ε-C_α-3′ (Fig. 11) (Honjo, 1983; Shimizu *et al.*, 1982). In humans, the sequence is 5′-C_μ-C_δ-$C_{\gamma 3}$-$C_{\gamma 1}$-$C_{\psi\varepsilon}$-$C_{\alpha 1}$-$C_{\gamma 2}$-$C_{\gamma 4}$-C_ε-$C_{\alpha 2}$-3′ on chromosome 14 (Honjo, 1983; Burrows and Cooper, 1984; Flanagan and Rabbitts, 1982). The precise mechanisms whereby B cells expressing surface IgM switch to downstream isotypes remain somewhat elusive. Currently, evidence is available for the occurrence of two switch pathways, i.e., either direct or successive isotype switches (Fig. 11). With direct switching, $sIgM^+$ B cells switch to expression of one of the downstream isotypes (e.g., $\mu \rightarrow \gamma 3$, $\mu \rightarrow \varepsilon$, and $\mu \rightarrow \alpha$). Alternatively, switches may occur linearly from 5′ → 3′ ($\mu \rightarrow \gamma$

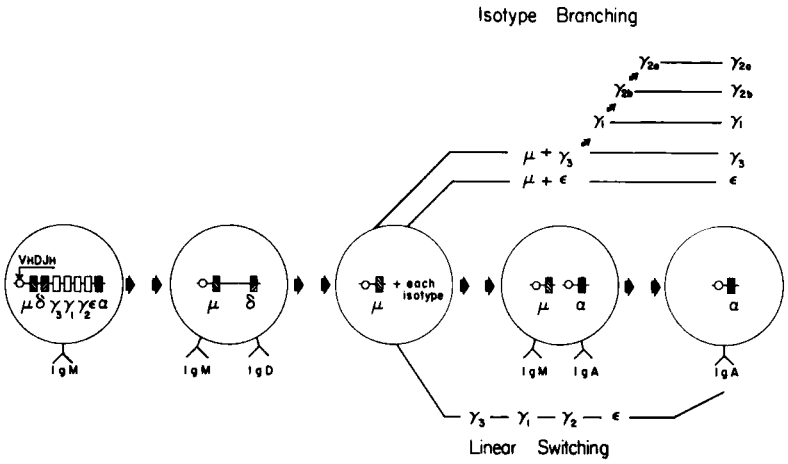

FIG. 11. Models for B cell switching that results in the expression of IgA on surfaces of B cells.

$\rightarrow \varepsilon \rightarrow \alpha$). While evidence exists for the occurrence of both direct and indirect isotype switching, there is still debate about the relative frequency with which these result in switches by normal B cells.

In direct switching to distant 3′ genes such as mouse ε or α, two basic processes have been proposed. In the first, a long primary mRNA transcript would encode VDJ-C_μ-C_δ and extend to, e.g., C_ε (Honjo, 1983; Shimizu and Honjo, 1984; Yaoita *et al.*, 1982). Differential mRNA splicing could result in VDJ-C_μ and VDJ-C_ε translated μ and ε chains and to subsequent membrane expression of IgM and IgE (Yaoita *et al.*, 1982). Alternatively, the C_μ and C_ε or C_μ and C_α genes could be brought together by DNA homology pairing of switch regions, and could subsequently result in formation of a shorter mRNA transcript required for 3′ isotype expression (Burrows and Cooper, 1984; Marcu and Cooper, 1982). The second model would require deletion of intervening DNA segments. Switches from C_μ to C_α, for example, would result in juxtaposition of C_μ and C_α genes, with the deletion of the entire C_γ genes and C_ε gene segments (Honjo, 1983; Honjo and Kataoka, 1978). Evidence from several systems that favor this latter mechanism have been presented. It is now clear that simultaneous RNA processing can occur for VDJ-C_μ and -C_δ. However, it has been difficult to demonstrate longer mRNA transcripts for other H chain switches.

The second major model proposed for H chain switches (stochastic model) involves linear 5′ → 3′ switches. This model was originally

proposed for B cells responding to TD antigens (Cebra *et al.*, 1979, 1982). The model is linear in the sense that B cells which have lost expression of the C_μ gene are committed to 3′ switching. This model of successive switching naturally assumes an irreversible process and would favor deletion of intervening C_H genes rather than looping out or sister chromatid exchange (Shimizu *et al.*, 1982; Honjo and Kataoka, 1978; Obata *et al.*, 1981). This switch process has been favored by Cebra and colleagues, and would help explain the high frequency of committed IgA B cells in GALT (Cebra *et al.*, 1984). This would assume that continued, successive B cell division through many generations would be accompanied by further downstream switching, ultimately leading to switches to α. This would occur especially in B cell germinal centers in GALT, where antigen/mitogen-driven B cell division is perhaps a major factor in switching. Thus, in the mouse the ultimate gene expressed would be the most 3′, i.e., α.

The role of T cells and antigen in B cell H chain switches remains controversial (see Section IV,E,2). Some investigators maintain that H chain switching, including commitment to IgA, occurs as a preprogrammed event (Cooper, 1981). Some evidence for switching has been presented in pre-B cells, and this of course would occur prior to the cell's responsiveness to either T cell influences or to antigen. The stochastic linear switching model envisioned by Cebra and co-workers (1984) would be influenced by antigen for B cell commitment to IgA. It would also help account for B cell switches that are induced by Tsw cells and derived switch factors.

In the discussion of mechanisms of class switching, including commitment to IgA, it is important to consider recent studies with rabbit C_H genes. The gene organization is approximately similar to that in mouse, i.e., VDJ-C_μ-C_γ-C_ε-3′ with C_α genes presumably residing 3′ of C_ε (Knight *et al.*, 1985). However, Knight and colleagues have made the striking observation that rabbits contain only one C_γ gene, unlike the four subclass genes described in humans and in mice. On the other hand, mice contain 1 α gene, humans contain 2 α genes (α1 and α2), while rabbits possess as many as 10 C_α genes (Honjo, 1983; Knight *et al.*, 1985). Although the complete organization of these C_α genes is not yet known, it appears that at least three of these are 3′ to C_ε (Knight *et al.*, 1985). It would be tempting to speculate that expression of these 3′ genes are unique characteristics of IgA committed B cells at mucosal inductive sites, e.g., the GALT. Of course, this cannot be tested in mice with only one C_α gene, but some evidence is available that the most 3′ human gene $C_{\alpha 2}$, which codes for IgA_2, has a clear association with mucosal immune responses. An association of

the three (or more) rabbit C_α genes which are the most 3′ with B cells committed to IgA in rabbit PP, would suggest that continued switches (and subsequent intervening C_H deletions) occur in the GALT.

7. *Oral Tolerance*

Ingestion of large doses of protein or continued ingestion of particulate antigens results in systemic unresponsiveness to the antigen, as demonstrated by Wells at the turn of this century (Wells, 1911). His studies showed that feeding ovalbumin (OVA) to guinea pigs resulted in their insensitivity to induction of systemic anaphylaxis, which we now know is caused by IgE- and IgG-subclass antibody responses. In 1946, Chase conducted studies which demonstrated that oral presentation of haptens, e.g., dinitrochlorobenzene, diminished contact hypersensitivity to these molecules (Chase, 1946). The use of other haptens that induced unresponsiveness after oral administration is commonly referred to as the Chase–Sulzberger phenomenon. The state of systemic unresponsiveness to orally administered haptens, proteins, and particulate antigens has now been termed *oral tolerance* (Tomasi, 1980). Interestingly, oral administration of antigen induces simultaneous responses, i.e., oral tolerance in systemic tissues and IgA responses at mucosal sites. It has been shown that oral administration of OVA or bacterial antigen induces both systemic unresponsiveness and salivary IgA responses (Challacombe and Tomasi, 1980).

The cellular and molecular bases of oral tolerance is still not clear; however, studies over the past 7 years have continued investigations of this problem. These studies have used three basic systems. As with the Chase–Sulzberger effect, studies of oral tolerance to contact hypersensitivity with picrylchloride, oxazolone, and similar agents have been performed (Asherson *et al.*, 1976; 1977a,b, 1978). A second system has involved oral administration of large doses (10–50 mg) of protein, e.g., ovalbumin (OVA) or bovine serum albumin (BSA), usually in mice to render them orally tolerant. Finally, prolonged oral administration of particulate antigens, usually SRBC to rodents, results in tolerance to this antigen when it is systemically administered. Just as three basic models have been used to study oral tolerance, three major mechanisms have been proposed to explain this effect. Andre *et al.* (1975) suggested that, in mice, systemic unresponsiveness to SRBC may be due to circulating immune complexes induced by the oral immunization regimen. This represented the first study which defined the precise conditions for induction of oral tolerance, and an analysis at the cellular level of the response (Andre *et al.*, 1975). Others (Kagnoff, 1980) have presented evidence to suggest that

anti-idiotypic antibodies may explain oral tolerance. Perhaps the major evidence to date suggests that oral antigen induces T suppressor (Ts) cells in GALT which subsequently home to systemic tissues such as spleen and peripheral lymph nodes and result in systemic unresponsiveness.

Extensive studies by Kagnoff *et al.* (1978a,b, 1980) have shown that continued feeding of SRBC to mice induces oral tolerance. Interestingly, serum but not cells of tolerant mice could transfer suppression to naive animals (Kagnoff, 1978b). This serum also effectively inhibited *in vitro* anti-SRBC antibody responses (Kagnoff, 1978b, 1980). The suppressive factor(s) in serum was apparently not due to circulating anti-SRBC antibodies, since absorption with antigen did not usually result in removal of suppressor activity (Kagnoff, 1978b, 1980). However, the factor(s) appeared to be immunoglobulin, since it could be removed by either protein A or anti-mouse Ig affinity column absorption (Kagnoff, 1978b, 1980). The ability to partially remove activity with antigens, and to totally remove suppression by removal of mouse Igs, probably indicates that the tolerance-inducing effect was due to the presence of anti-idiotypic antibodies (Kagnoff, 1980). However, the presence of antigen-specific Ts cell factors cannot be completely ruled out.

In a contact hypersensitivity system, Asherson *et al.* (1977b, 1979) have shown that two types of suppressor cells are induced by hapten. The first appears to be typical Ts cells which inhibit hapten-induced proliferative responses *in vitro* (Asherson *et al.*, 1977b, 1979). The second cell type, namely B suppressor cells, also inhibited the functional activity of T cell-mediated immunity responsible for the *in vivo* hypersensitivity reaction, when preincubated with these cells prior to adoptive transfer (Asherson *et al.*, 1977b, 1979). Numerous studies have shown that Ts cells can induce this immune function (see below). Studies with soluble proteins have also indicated that Ts cells are induced in PP (Richman *et al.*, 1981) and are present in secondary lymphoid tissues such as spleen (Richman *et al.*, 1978; Miller and Hanson, 1979). Adoptive transfer of these Ts cells to normal mice renders the animal unresponsive to systemic antigen (Richman *et al.*, 1981, 1978; Miller and Hanson, 1979). It is important to emphasize that in some studies, oral administration of antigen also induces development of Th cells in PP (Richman *et al.*, 1981) and helper factors in spleen cell cultures (Germain and Benacerraf, 1981). Generally, the Ts cell induced is Lyt-1^-,2^+ with general characteristics of Ts cells that mediate classical suppression (Mattingly and Waksman, 1980).

Studies with the SRBC system have shown that continued feeding

or gastric intubation with SRBC results in the presence of T cells with characteristics of the suppressor phenotype in both rats (Mattingly and Waksman, 1978) and mice (MacDonald, 1982a,b). In this regard, it was shown that spleen cell cultures from mice (Mattingly *et al.*, 1980) or rats (Germain and Benacerraf, 1981) orally tolerized with SRBC released factors which mediate suppression. Evidence for Ts cell involvement in oral tolerance is also strongly suggested by studies with high IgA responder C3H/HeJ (Lps^d/Lps^d) and syngenic C3H/HeN mice (Lps^n/Lps^n). Prolonged oral immunization of C3H/HeN mice with SRBC leads to the induction of systemic unresponsiveness to intraperitoneal (IP) challenge with SRBC–antigen. On the other hand, oral tolerance to SRBC cannot be induced in C3H/HeJ mice. Instead, when given SRBC by IP injection, after prolonged gastic intubation with SRBC, these mice elicit good secondary responses of the IgM, IgG, and mainly IgA isotypes (Kiyono *et al.*, 1982c; Michalek *et al.*, 1982).

Extensive analysis of the regulatory T cells in PP and spleens of orally tolerized C3H/HeN mice showed a predominant Ts cell activity (Fig. 12). In contrast, a predominant Th cell pathway was noted in

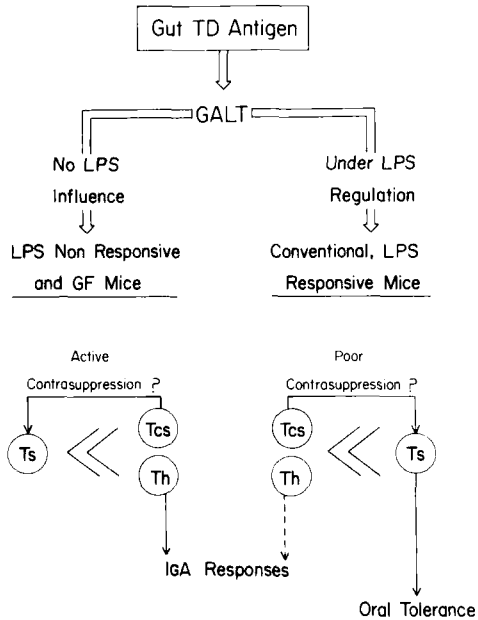

FIG. 12. Proposed mechanisms for the role of intestinal lipopolysaccharide (LPS) in the regulation of oral tolerance and IgA immune response by T cells.

both spleen and PP of C3H/HeJ mice (Kiyono *et al.*, 1982c; Michalek *et al.*, 1982). Recent studies suggested that other regulatory T cells, e.g., contrasuppressor T cells (Tcs), are involved in induction and regulation of oral tolerance and secondary immune responses. Adoptive transfer of Lyt-1^+,2^-, I-J^+, and *Vicia villosa* adherent Tcs cells from orally immunized C3H/HeJ mice to orally tolerant C3H/HeN mice resulted in complete abrogation of oral tolerance (Suzuki *et al.*, 1986b). Furthermore, adoptive transfer of PP Tcs cells from C3H/HeJ mice induced a predominant IgA response in C3H/HeN mice that were rendered tolerant (Suzuki *et al.*, 1986a). These results again confirmed that oral tolerance to SRBC is largely mediated by a Ts cell network. In addition, one may explain induction of high IgA responses and lack of oral tolerance induction in C3H/HeJ mice by the existence of an enhanced Tcs cell network in this strain of mice (Fig. 12). It could be further suggested that these effector Tcs cells have their origin in GALT, and that Tcs cells regulate both systemic unresponsiveness and IgA responses at distant mucosal sites (Fig. 12).

As indicated above, much additional work will be required to determine the precise mechanisms which regulate host unresponsiveness to oral antigens. It is now clear that Ts cells play a major role in the regulation of systemic unresponsiveness to orally encountered antigens. Generally, down-regulation of immune responses is considered to be the major function of the Ts cell network (Mattingly and Waksman, 1980). This consists of three interacting T cell types. The first Ts cell exhibits antigen specificity and is termed a T_{S1} cell type. The second member of the network, or T_{S2} cell, has idiotype (ID) specificity, while the third, or T_{S3} cell, has anti-ID_2 or antigen specificity (Mattingly and Waksman, 1980). Factors produced by each cell type are identical to the specificity exhibited by the Ts cell (Mattingly and Waksman, 1980). These antigen- and idiotype-specific Ts cells and specific factors interact to induce antigen-specific immunosuppression. One would expect that Ts cells induced by oral antigen would also consist of this tripartite circuit, and studies should be directed to determine whether this is indeed the case. Furthermore, a need exists to determine how contrasuppressor cells function at IgA inductive sites, in order to allow the simultaneous induction of mucosal IgA responses with concomitant induction of oral tolerance (Challacombe and Tomasi, 1980).

V. Concluding Remarks

Most immunologists have considered IgA primarily as an immunoglobulin isotype characteristic of external secretions from many

mammalian species. Recent studies suggest that an immunoglobulin isotype homologous with mammalian IgA may have evolved in amphibians (Hadge and Ambrosius, 1984), thus making IgA the phylogenetically second oldest immunoglobulin isotype. In many animal species and in humans (Delacroix, 1985) the daily production of IgA far exceeds production of all other immunoglobulin isotypes. The principal organs involved in the production of such large quantities of IgA are the intestines and bone marrow. In this article we outlined the unique regulatory principles of cellular biosynthesis of IgA by isotype-specific T cells. However, the mechanisms responsible for selective distribution of polymeric and monomeric IgA forms in different body fluids depend upon interactions of these molecular forms with various populations of nonlymphoid cells. A major unresolved problem in IgA physiology concerns the fate of large quantities of circulating monomeric IgA and its function. Although the protective role of S-IgA has been well established the function of monomeric serum IgA remains an enigma.

The inability of IgA to bind complement effectively and promote phagocytosis may be of advantage in the noninflammatory disposal of foreign antigens from mucosal surfaces. Serum IgA may shield endogenous antigens from the potentially harmful effects of complement-binding and phagocytosis-promoting autoantibodies of IgG and IgM isotypes often found in patients with IgA deficiency (Mestecky *et al.*, 1986b). Further explorations will be necessary to establish the validity of this concept.

To achieve protection of large surfaces of mucous membranes against infectious agents and environmental antigens by selectively inducing S-IgA antibodies will require novel immunization approaches with respect to the dose, frequency of immunization, and route of delivery of antigens. Elucidation of the origin, site of sensitization with antigen, and migratory pathways of cells that produce polymeric IgA in secretory glands and tissues and its selective transport into external secretions led to the formulation of the concept of a common mucosal immune system. Ingestion or inhalation of antigens that sensitize cells in the anatomically unique mucosa-associated lymphoid tissues is followed by dissemination of the antigen-sensitized IgA precursor cells that home to several secretory glands and mucosa-associated tissues. Local differentiation into plasma cells and the selective transport of the product results in the simultaneous appearance of specific antibodies at several sites. Accumulated experimental evidence confirming the validity of this concept strongly suggests that the design of future vaccines that will prevent the entry of

infectious agents through mucosal surfaces may be based on the knowledge gained from studies of the cycle of IgA cells. Unique antigen delivery systems that would take advantage of genetically engineered bacteria that selectively colonize GALT or BALT, for a limited period of time, and deliver cloned gene products from other microorganisms into these lymphoid organs may be utilized (Keren *et al.*, 1982; Curtiss *et al.*, 1983,1986). Alternatively, some antigens chemically linked to a substance such as B subunit of cholera toxin may also be used for the targeted delivery of desired antigens. Novel adjuvants, usable in oral immunization and antigen packaging, may further increase the effectiveness of such orally administered vaccines against various human and veterinary infectious diseases. Additional potentiation of vaccine effectiveness and selectivity of antibody isotype induced may be achieved through the use of products of IgA isotype-specific regulatory T cells.

Addendum: Recently, two subsequent communications appeared in *Immunology Today*, Vol. 7, p. 174 and p. 206 (1986), which deal with computer models of the human immunoglobulins. These articles, written by R. Pumphrey, used computer graphics and currently available sequence and crystallographic data to predict the arrangements of domains and polypeptide chains in immunoglobulins including secretory component, J chain, monomeric and polymeric IgA, and secretory IgA. The reader is referred to these two excellent articles for details of the models predicted. Recombinant murine BSF_1, now termed IL-4, shares several properties including BCGF I activity and induction of LPS-treated B cells to differentiate into IgG_1 and IgE plasma cells [Noma *et al.* (1986), *Nature* (*Lond.*) **319**, 640]. Cloned BSF_2, formerly BCDF or TRF, has been named IL-5 and exhibits both BCDF/TRF and BCGF II activity [Kinashi *et al.* (1986), *Nature* (*Lond.*) **324**, 70].

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ADVANCES IN IMMUNOLOGY, VOL. 40

The Arrangement of Immunoglobulin and T Cell Receptor Genes in Human Lymphoproliferative Disorders

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I. Introduction

The human immune system has a virtually unlimited capacity to recognize and distinguish specific molecular patterns and thus to bind millions of potential antigens. In normal individuals, this is accomplished with an array of tissue and circulating B and T lymphocytes that represent a complex mixture of different subsets of cells with different and, at times, opposing functions. A number of the insights concerning this network of cells have emerged from the study of clonal neoplasms of the B cell/plasma cell and T cell series. For example, the recognition that paraproteins derived from patients with multiple myeloma represent homogeneous immunoglobulins was an indispensable step in understanding the structural and functional aspects of immunoglobulin molecules and antibodies. As new techniques have been developed, they have been applied to the study of malignant lymphocyte populations to provide insights concerning the functions of the normal immune system, as well as to provide answers of interest to the clinicians caring for patients. Historically, the diagnosis of hematopoietic malignancies was based on the appearance of abnormal-looking cells in the lymph nodes, bone marrow, peripheral blood, or other tissues which gave rise to classifications as lymphocytic, monocytic, or histiocytic leukemias or, when the cell type could not be defined, to such purely descriptive terms as hairy cell leukemia. Subsequently, heteroantisera and then monoclonal antibodies that identify distinct surface determinants that make their appearance on lymphoid cells as they mature and differentiate have proved valuable in the diagnosis and classification of lymphoid neoplasms. Although the ability to distinguish clonal B and T cell subpopulations by surface phenotype analysis was a major advance, monoclonal antibody studies are not always conclusive. For example, the results of such studies may be ambiguous when the tissue sample contains admixtures of normal and neoplastic cells. This approach is also not adequate to define the clonality of lymphocyte populations. Furthermore,

although there have been correlations between the surface phenotype and the function of the cells, in the majority of cases most monoclonal antibodies used do not define antigens intimately related to the function being considered, and thus cells with different functions, for example, adult T cell leukemia (ATL) cells and Sézary cells, may share a common phenotype (i.e., CD3/CD4 [T3, T4] positive and CD8 [T8] negative). To address this last issue, a series of techniques have been developed to analyze the retained functions of T cell leukemic cells. These approaches have allowed scientists to define different T cell leukemias as proliferations of helper, prosuppressor, suppressor–inducer, or suppressor–effector cells. These approaches were reviewed previously in this series (Waldmann and Broder, 1982). Recently, there have been dramatic advances in our understanding of the vertebrate immune system that have followed the application of recombinant DNA technology to the study of mouse and human malignancies of the B lymphocyte/plasma cell and T lymphocyte lineages. An analysis of the arrangement of immunoglobulin and T cell receptor genes and the rearrangement of these genes as B and T cells mature has provided answers for the two most critical questions that immunologists have addressed over the past two decades: what are the mechanisms used to generate antibody diversity, and what is the nature of the antigen-specific T cell receptor? It has been shown that the genes ultimately specifying the structure of each antibody or T cell receptor are not present as such in germ cells (Leder, 1982; Honjo, 1983; Tonegawa, 1983; Hedrick *et al.*, 1984a,b; Hood *et al.*, 1984; Yanagi *et al.*, 1984). Rather, they are organized as discontinuous DNA segments in their germ-line form, the form present in virtually all of the nonlymphoid cells of our body. However, as an uncommitted bone marrow stem cell differentiates into an antibody-synthesizing plasma cell, an orchestrated series of immunoglobulin gene rearrangements occurs that serves to activate these genes and to increase the diversity of the gene products (Hozumi and Tonegawa, 1976; Brack *et al.*, 1978; Rabbitts and Forster, 1978; Seidman and Leder, 1978; Seidman *et al.*, 1978; Hieter *et al.*, 1980; Kataoka *et al.*, 1980; Leder *et al.*, 1980; Sakano *et al.*, 1980; Leder, 1982; Honjo, 1983; Tonegawa 1983). Similarly, for the T cell, the genes encoding the antigen receptor are shuffled as precursor cells develop and mature into T lymphocytes (Hedrick *et al.*, 1984a; Yanagi *et al.*, 1984). The rearrangement of these gene segments can lead to millions of different results in the cells of these two lineages. For B cells, individual mutations amplify the diversity. The result is that, in the mature descendants of each line, a

unique gene is assembled whose information is expressed in the form of a unique antibody or a unique T cell receptor.

The rules of DNA rearrangement and transcriptional regulation that have been learned by studying B and T cell tumors have proved of great value to the hematologist/oncologist in dealing with patients with lymphoid neoplasms. The analysis of rearranged immunoglobulin and T cell receptor genes has been of value in (1) defining of the origin of neoplasms that were of controversial lineage previously; (2) determining whether abnormal lymphocytic proliferations were monoclonal, oligoclonal, or polyclonal; (3) diagnosing, defining the extent, and monitoring the therapy of lymphoid malignancies; (4) determining the state of maturation and the causes for failure of maturation of cells of the B and T cell precursor series; (5) obtaining insight into the cause of the malignant transformation of these cells by the analysis of translocations of cellular transforming genes into the sites of immunoglobulin or T cell receptor genes; and (6) defining the pathogenesis of certain hereditary immunodeficiency diseases (Korsmeyer *et al.*, 1983a; Korsmeyer and Waldmann, 1984; Cleary *et al.*, 1984a; Sklar *et al.*, 1984; Waldmann *et al.*, 1985b). The present article will focus on the insights that have been obtained by applying molecular genetic analyses of the structure and arrangement of immunoglobulin and T cell receptor genes to the study of human lymphoid proliferations.

II. Somatic Rearrangement of Immunoglobulin Gene Elements Creates a Functional Antibody Gene

A. Human κ Light Chain Genes

An individual immunoglobulin is comprised of two identical light and two identical heavy chain molecules that are encoded in three unlinked gene families: κ light chain genes, λ light chain genes, and heavy chain genes. Of the two available light chains, κ light chains comprise approximately 60% of human light chain protein. During the early development of a B cell that will ultimately produce a κ light chain, a DNA rearrangement occurs in the κ locus to create an active gene. The structure of the genes of this locus is presented in the schematic diagram in Fig. 1 (Hieter *et al.*, 1980). The human κ gene locus is located on chromosome 2 at band 2p13 (Hieter *et al.*, 1980; Malcolm *et al.*, 1982; McBride *et al.*, 1982). The first 95 amino acids of the final κ variable region protein are encoded by multiple V_κ seg-

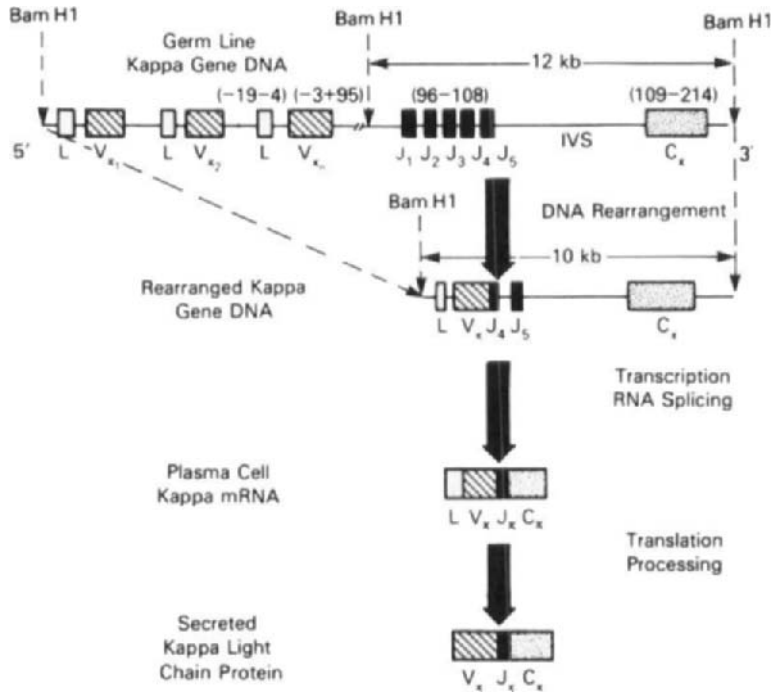

FIG. 1. Arrangement of human κ gene locus. Multiple variable (V_κ) regions exist, each with an associated leader (L) sequence. There are five alternative joining (J_κ) segments encoding the remainder of the variable region. There is a single constant (C_κ) region element per allele. DNA rearrangements lead to the juncture of a single V_κ with one of the five J_κ segments. When the gene is activated, RNA is transcribed and the intervening sequences (IVS) are removed by RNA splicing. The rearrangement that juxtaposes a single V_κ with a J_κ element results in a change in the location of a *Bam*HI restriction endonuclease site. This event alters the size of the restriction fragment that bears the C_κ gene element and allows the allele to be distinguished from the germ-line configuration by Southern analysis.

ments. Each of these V_κ segments has two exons separated by an intron. The 5′ exon codes for a major portion (residues −19 to −4) of the leader sequence, a highly hydrophobic peptide that facilitates transmembrane passage of the molecule and that is eventually cleaved off. The 3′ V_κ exon codes for the remaining portion of the leader sequence and the major portion of the variable region (residues −3 to 95). The remainder of the variable region of the κ chain is encoded by one of five functional J_κ sequences. There is a single constant κ (C_κ) region gene separated from the J_κ region by a long intervening sequence. At some point during the differentiation of a

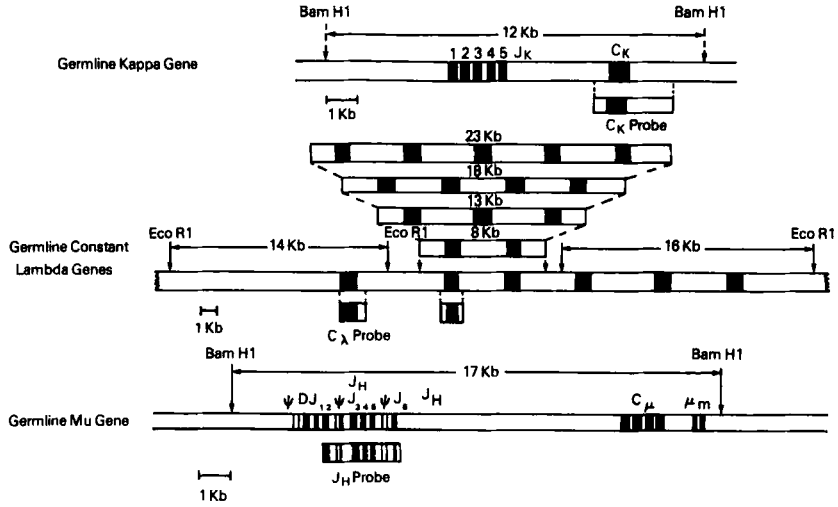

FIG. 2. Schematic representation of the germ-line κ, λ, and heavy chain loci indicating the gene segments most commonly used as probes. The human constant λ (C_λ) region in its most contracted form has six constant regions, each with its associated J region (J not shown) that are on 14-, 8-, and 16-kb *Eco*RI restriction fragments. Multiple allelic forms of the central *Eco*RI fragment exist which can be 8, 13, 18, or 23 kb in size. Each incremental enlargement of this central fragment increases the number of available J_λ and C_λ genes. The heavy chain locus includes six functional joining (J) regions, a D segment (D), and three pseudo J genes (ψ). Both secreted and membrane forms of IgM are derived from a single constant μ region locus that includes four C_μ domains. Alternative sites of poly(A) addition and RNA splicing result in different mRNAs containing either the secreted or the hydrophobic membrane ($C_{\mu m}$) terminus. For the $C_{\mu m}$ terminus, two additional exons ($C_{\mu m}$) are utilized. The J_H probe identifies a germ-line 17-kb *Bam*HI band. A J_H rather than C_μ probe is utilized in the analysis, since the C_μ region may be deleted during immunoglobulin class switch.

pluripotent stem cell into a terminally differentiated κ-producing plasma cell, a process of DNA rearrangement occurs that joins one of the many germ-line variable (V_κ) regions with a particular joining (J_κ) region. This rearranged allele is transcribed, and the intervening sequences are removed by RNA splicing. The final mature mRNA is then translated into the complete κ light chain protein.

B. λ LIGHT CHAIN GENES

λ light chains are present in approximately 40% of human immunoglobulin molecules, whereas they are present in less than 5% of the immunoglobulins of the mouse. The λ locus is organized in a slightly different fashion from the other immunoglobulin loci (Fig. 2). It is composed of multiple, different C_λ regions, each associated with

its own J_λ segment (Blomberg *et al.*, 1981; Hieter *et al.*, 1981a; Taub *et al.*, 1983). The majority of human λ genes are arranged in tandem along the long arm of chromosome 22 at band 22q11 (McBride *et al.*, 1982). Nucleic acid sequencing of the first three constant regions of this locus has shown that they represent genes encoding the C_λ regions bearing the amino acid markers Mcg, Ke^-Oz^-, and Ke^-Oz^+, respectively, isotypes that were previously identified by serological and amino acid analysis of human λ light chain proteins (Fig. 2). The human λ gene locus has been proved to be rather polymorphic in the normal population as evidenced by a restriction fragment-length polymorphism (RFLP). A DNA RFLP is produced by a restriction endonuclease that is an enzyme that recognizes a specific base pair sequence and reproducibly cuts DNA only where this site appears. A RFLP is apparent when there is a genetically transmitted variation in the base sequence identified by the restriction endonuclease used. The genetic polymorphism in the λ locus is manifested as a variation in the length of the DNA fragment that composes the central portion of this locus, when digested with the restriction endonuclease *Eco*RI. Taub *et al.* (1983) reported that in its simplest form this fragment is 8 kb in size and contains the two constant λ regions Ke^-Oz^- and Ke^-Oz^+. There are, however, multiple allelic forms of this center fragment which can be 8, 13, 18, or 23 kb in size. The incremental enlargement of this central *Eco*RI fragment is a series of 5-kb additions, each fragment containing a single C_λ region. Thus, the 8-kb allelic fragment has only two C_λ regions, whereas the 23-kb alternative possesses five C_κ regions on this central fragment. Thus, the actual number of C_λ genes in humans can vary between six and nine on this portion of chromosome 22 depending on whether the 8-, 13-, 18-, or 23-kb allele is inherited. Thus, in contrast to the κ genes that use a tandem strip of multiple J segments, the λ gene locus has expanded by amplifying $J_\lambda C_\lambda$ units.

C. Heavy Chain Gene Assembly

The human heavy chain gene locus is on chromosome 14 at band 14q32 (Croce *et al.*, 1979; Kirsch *et al.*, 1982). While the general scheme of heavy chain gene organization is similar to that of light chain genes, it has the additional complexity of a third component of the variable region known as the diversity segment (D_H) that enables heavy chains to make an even greater contribution to the generation of antibody diversity (Early *et al.*, 1980a; Sakano *et al.*, 1980, 1981; Ravetch *et al.*, 1981; Siebenlist *et al.*, 1981) (Fig. 2). This short D_H segment, encoding a variable number of amino acids, appears in

closely related families that are arranged in tandem within the genome between the V_H and J_H segments. The major portion of the variable region domain of heavy chains is contributed by a series of variable (V_H) region gene segments that encode approximately the first 99 amino acids of the variable portion of the molecule, including the first and second hypervariable regions (CDR1 and CDR2). There are six active joining (J_H) region gene segments in man that are located 5′ to the constant region genes. Additionally, there are three pseudo J_H sequences that exist in this area, as does one germ-line D_H element. In order to activate and correctly assemble a heavy chain gene, one of the multiple D_H region genes must correctly rearrange and join with one of the six functional J_H segments, and then one of the few hundred V_H region gene segments must join this DJ juncture. While the heavy chain gene locus is rich in recombinational opportunities, it appears that the chances of assembling all these segments correctly is low, with nonproductive recombinations of these segments (V_H, D_H, and J_H) occurring in over 80% of recombinations.

D. Recombinational Mechanisms Involved in Joining the Segments of the Variable Region

The mechanisms that lead to the joining of the segments of the variable region have not been fully defined. However, DNA sequences that appear to serve as signals for the joining of variable, diversity, and joining segments have been identified (Max *et al.*, 1979, 1980; Sakano *et al.*, 1979, 1981; Seidman *et al.*, 1979; Hood *et al.*, 1984). The separate DNA segments encoding the variable regions of immunoglobulin genes are flanked by conserved heptamer and nonamer sequences separated by nonconserved spacer DNA that may be either 11, 12, or 23 ± 1 nucleotides long (Fig. 3). Each of the germ-line V, J, and D subsegments is flanked by a heptanucleotide (which is an inverted repeat or palindrome of CAC$\frac{A}{T}$GTG). This heptanucleotide is found on the 3′ side of each V segment, the 5′ side of each J segment, and on both sides of the D_H segment. Flanking each heptanucleotide of a V_H, J_H, J_κ, and V_λ is a spacer of 22 or 23 nucleotides and then a stretch of 9 conserved base pairs. This 9 base pair (9-bp) segment is complementary to a nonanucleotide that is separated by 11 or 12 bases from a heptanucleotide flanking the germ-line D_H, V_κ, and J_λ segments. Note that the heptameric and nonameric sequences following a gene segment (for example, V_κ, V_λ, V_H, or D_H) are complementary to those preceding the gene segment (in this case, J_κ, J_λ, D_H, and J_H, respectively) with which they combine. Furthermore, it appears that a heptanucleotide and nonanucleotide possessing an 11- or 12-bp

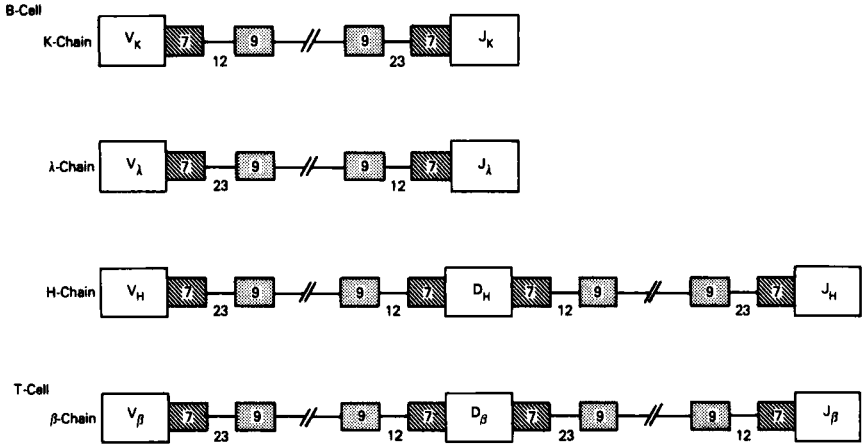

FIG. 3. Immunoglobulin and T cell receptor genes are flanked by conserved heptanucleotide and nonanucleotide sequences separated by nonconserved spacer DNA that may be 12 or 23 nucleotides long. The heptamers and nonamers 3′ to a segment (e.g., V or D) are complementary to the nonamers and heptamers 5′ to a downstream segment (e.g., D or J). A heptanucleotide and nonanucleotide possessing an 11- or 12-bp spacer always pairs with a complementary set of sequences containing a 23 ± 1-bp spacer. According to this 12/23-bp rule, the joining of two immunoglobulin D heavy chain segments or the direct joining of an immunoglobulin V_H with a J_H segment would not be possible. In contrast, in the T cell β gene system, the D region is flanked by a 5′ 12-bp and a 3′ 23-bp spacer, therefore D_β/D_β and V_β/J_β joinings would theoretically be possible.

spacer always pairs with a matched set of sequences containing the 23 ± 1-bp spacer. According to this 12/23-bp rule, the joining of two D heavy chains or the joining of a V_H and a J_H would not be possible. Since one turn of the DNA helix requires approximately 10.4 bp, the conserved spacing of 12 or 23 bp corresponds to either one or two turns of the helix. The two blocks of conserved nucleotides (nonamer and heptamer) are thus located on the same side of the helix and may provide the site of action of postulated recombinases. The fact that the heptanucleotides, nonanucleotides, and their spacers flank all of these immunoglobulin gene segments and are found in all species examined suggests that they are active participants in bringing V_L/J_L and $V_H/D_H/J_H$ regions together. One possible model for the mechanism of joining of two segments is that the two complementary heptanucleotides and the two complementary nonanucleotides would base pair, creating a stemlike structure that would facilitate recombination between the strands. The stem and loop structure would be deleted by enzymatic action. This model predicts that any flanking DNA lo-

cated between the juxtaposed V_κ and J_κ segments would be deleted from the genome during this process of DNA rearrangement. In a number of cases the intervening DNA segment between V and J segments was shown to be deleted in myelomas, an observation that is consistent with the stem and loop structural model (Seidman *et al.*, 1980). However, it has subsequently been observed that DNA segments 5′ to the J_κ segment were retained in some myelomas that had rearranged both J_κ gene segments (Höchtl *et al.*, 1982; Lewis *et al.*, 1982; Van Ness *et al.*, 1982). To explain this observation, it is proposed that alternative mechanisms may occur in some cases, including more complicated genetic crossovers or inversion for gene construction. Direct evidence for chromosomal inversion has been presented for the T cell receptor β gene which has a V_β element mapped to the 3′ side of the $C_{\beta 2}$ gene in an inverted transcriptional polarity relative to the D_β, J_β, or C_β genes (Malissen *et al.*, 1986).

The genetic rearrangements that lead to V gene formation do not occur at a precise location relative to the recognition sequences (Bernard *et al.*, 1978; Max *et al.*, 1980). This flexibility at the joining region provides alternate triplet codons and thus additional amino acids at this ninety-sixth position of the κ light chain. The ninety-sixth amino acid importantly resides within the third hypervariable region and thus may create amino acid substitutions that contribute to antibody diversity. The imprecision of joining of V_H, D_H, and J_H segments is even more striking with variability extending over at least 10 nucleotides. Although flexibility of joining provides an additional mechanism for antibody diversity, it also leads to nonproductive rearrangements. Because all immonoglobulin gene segments appear to be translated in only one reading frame in order to produce a functional immunoglobulin chain, one implication of the imprecision is that the gene segments often join in an out-of-phase reading frame. This nonproductive joining occurs frequently in lymphoid cells. Thus, the additional diversity-generating mechanism is achieved at the expense of some cell waste.

Another mechanism that leads to junctional diversity appears to be the addition of one or more nucleotides at the junction of V_H, D_H, and J_H segments (Alt *et al.*, 1982; Desiderio *et al.*, 1984). When comparisons were made of germ-line and somatic D sequences, one to several nucleotides at both ends of many somatic D sequences were present that could not be explained by the apparently corresponding germline D segments. Interestingly, these extranucleotide sequences, termed the "N" region, are often a run of Gs or Cs. Based on these findings, Alt and Baltimore (1982) have suggested that these nucleo-

tides may be added at the ends in a template-free fashion, perhaps by the enzyme terminal deoxynucleotidyl transferase.

An additional mechanism contributing to antibody diversity is the occurrence of point mutations that result in changes in amino acids including those within the variable region of the peptide (Bothwell *et al.*, 1981; Gearhart *et al.*, 1981; Kim *et al.*, 1981). The demonstration of alterations in the variable regions of immunoglobulin light and heavy chain proteins, when compared with the parental germ-line variable region genes, provided evidence supporting somatic mutation. The frequency of mutation appears to be very high. The regions in which hypermutation occurs are highly restricted to the VJ or VDJ segments and to the few hundred base pair untranslated regions surrounding these segments but do not involve the constant segment genes for the immunoglobulin light or heavy chains. It was initially suggested that they may be linked to heavy chain switch recombination; however, it has also been proposed that these mutations occur in association with VJ and VDJ joining.

E. Sequential Activation of Immunoglobulin Genes

A sequential activation of immunoglobulin variable region genes has been demonstrated by Alt *et al.* (1980) studying Abelson-transformed B cells of the mouse and by Korsmeyer, Hieter, and their coworkers (Hieter *et al.*, 1981b; Korsmeyer *et al.*, 1981) examining immunoglobulin gene rearrangements in human leukemic B cells and B cell precursors. The initial event in immunoglobulin gene recombination involves the junction of the D_H and J_H segments of the heavy chain gene (Alt *et al.*, 1984). Subsequently, a V_H gene segment is combined with this DJ juncture. This initial attempt at recombining a V_H, D_H, and J_H segment may be effective, in which case cytoplasmic μ chain may be produced or may be aberrant with no complete heavy chain synthesized. Alt *et al.* (1982) proposed that a productive $V_H/D_H/J_H$ rearrangement leading to the synthesis of a heavy chain polypeptide inhibits further heavy chain gene rearrangements. Weaver *et al.* (1985) have provided evidence in support of this model of active prevention of rearrangement by the product of successfully rearranged μ genes. In these studies, microinjection of a rearranged immunoglobulin μ heavy chain gene into transgenic mice prevented the rearrangement of endogenous heavy chain genes. Following effective heavy chain gene rearrangement, the first attempt at light chain gene rearrangement involves the κ gene class. If a V_κ and a J_κ gene segment are effectively joined in a cell already possessing an effective $V_H/D_H/J_H$ recombination, a μ, κ surface immunoglobulin B cell would result.

Frequently, however, both the maternal and paternal set of κ alleles aberrantly rearrange or are even deleted. If both κ genes have rearranged nonproductively, λ gene rearrangements are initiated and, if effective, result in a μ,λ-bearing B cell. If such rearrangements are also aberrant, the cell may be retained in the pre-B cell series, not capable of producing light chains. Thus, there is a hierarchy of immunoglobulin gene recombinations that moves from heavy chain genes to κ light chain genes and then to λ light chain genes. This cascade of gene rearrangements is quite error prone. Thus, a sizeable proportion of B cell precursors entering this pathway may waste all chance of forming an effective heavy or light chain variable region gene and may be incapable of further expansion.

F. Heavy Chain Constant Region Gene Order and Class Switching

Following the rearrangement of heavy and light chain gene variable segments, a B cell and its progeny will produce only a single variable heavy chain domain and a single light chain variable region. Furthermore, the cell will produce only a single form of light chain, κ or λ, but not both. However, a B cell is capable of simultaneously producing IgM and IgD membrane forms of immunoglobulin and is capable of switching subsequently to the production of IgG, IgA, or IgE (Cory *et al.*, 1980b; Davis *et al.*, 1980; Early *et al.*, 1980b; Ravetch *et al.*, 1980; Kataoka *et al.*, 1981; Maki *et al.*, 1981; Moore *et al.*, 1981; Obata *et al.*, 1981; Flanagan and Rabbitts, 1982; Marcu *et al.*, 1982; Max *et al.*, 1982; Takahashi *et al.*, 1982). In addition, both membrane and secreted forms of these immunoglobulins may be produced by the same cell at different stages of differentiation (Early *et al.*, 1980b). Each of these heavy chain classes is associated with the same variable heavy chain region for a given cell. Establishing the order and structure of the heavy chain constant region genes has helped elucidate the mechanisms by which these different classes are produced. The heavy chain genes are in a cluster in a 200-kb region 5′ to the J sequences. The order of the mouse C_H genes is 5′-C_μ-C_δ-$C_{\gamma 3}$-$C_{\gamma 1}$-$C_{\gamma 2b}$-$C_{\gamma 2a}$-C_ε-C_α-3′ (Shimizu *et al.*, 1981). The human immunoglobulin heavy chain constant region genes located on the long arm of chromosome 14 at band q32 are in order 5′-C_μ-C_δ-$C_{\gamma 3}$-$C_{\gamma 1}$-$C_{(\text{pseudo})\varepsilon}$-$C_{\alpha 1}$-$C_{(\text{pseudo})\gamma}$-$C_{\gamma 2}$-$C_{\gamma 4}$-$C_\varepsilon$-$C_{\alpha 2}$-3′. During the differentiation of a single B lymphocyte, a given V_H gene is first expressed in combination with the C_μ gene of the same allelic chromosome, and later in the B lymphocyte C_μ and C_δ are expressed. Subsequently, by a heavy chain class switch, the same V_H gene is expressed in combination with a different C_H gene. The close

proximity of the C_μ and C_δ regions allows for the simultaneous production of IgM and IgD which bear the same assembled variable region (Knapp *et al.*, 1982). Nuclear RNA transcripts from a recombined $V_H/D_H/J_H$ region may undergo differential processing at alternate RNA splice sites to connect a single variable region gene with either the C_μ or C_δ constant region (Moore *et al.*, 1981). B $\mu\delta$ cells can differentiate and begin to synthesize other classes of antibodies. Such class switches occur by genetic recombination between switch regions that lie from 2 to 3 kb 5′ from each C_H gene, with the exception of the C_δ gene. These switch regions are large segments composed of multiple copies of short repeated elements. The repetitive nature of these switch regions may promote homologous recombinations between the μ switch region and further 3′ switch regions. Such recombinations would result in a DNA rearrangement that may be accompanied by deletion of the DNA between the switch region 5′ from the $C\mu$ gene and that switch region immediately 5′ from the constant region to be used. This process of switching allows for a new constant region to be transcribed with the preexisting $V_H/D_H/J_H$ recombined gene.

III. The T Cell Antigen Receptor Structure and Gene Organization

A. The Structure of the T Cell Antigen Receptor

The nature of the T cell receptor, the surface structure by which T cells recognize specific antigens and MHC, has been defined by a number of groups that used a similar approach (Allison *et al.*, 1982; Haskins *et al.*, 1983; Kappler *et al.*, 1983; Meuer *et al.*, 1983; Samelson *et al.*, 1983; Acuto and Reinherz, 1985). Each of the groups prepared monoclonal antibodies against lines of cloned T cells and looked for clonotypic antibodies that reacted only with the clone of cells used to elicit the antibody production. Furthermore, where functional lines were examined, they defined antibodies that inhibited the functional activity of the clone. When these antibodies were used under nonreducing conditions, they precipitated the T cell receptor that appeared as a single band at approximately 90,000 Da. Under reducing conditions, this structure from human cells was separated into two components of approximately 40 and 50 kDa. The larger and more acidic glycoprotein was termed the α chain, while the smaller and more basic was termed the β chain. Both the α and β chains of this polymorphic heterodimer were composed of a constant and variable domain. The heterodimer formed by these two chains is part of a macromolec-

ular complex that also includes the three or more invariant polypeptides recognized by anti-CD3 monoclonal antibodies (T3, Leu-4). The CD3 glycoprotein family is composed of three or more nonpolymorphic peptide chains of M_r 19,000, 21,000–23,000, and 25,000–28,000 (Borst *et al.*, 1983). The heterodimer of α and β chains appears to subserve the recognition function of the molecule. The CD3 complex, which is noncovalently associated with the heterodimer, appears to be responsible for transducing the signal to the interior of the cell and initiating the cellular response to antigen. The interaction of appropriately presented antigen, lectins, or monoclonal antibodies with the T cell antigen receptor complex leads to the generation of inositol triphosphate (IP_3), an increase in the concentration of cytoplasmic-free calcium $[Ca^{2+}]_i$, and an activation of protein kinase C (Weiss and Stobo, 1984). These events lead to the initiation of second stage events in which previously untranscribed genes become expressed: specifically, the genes encoding the lymphokine IL-2 and those encoding the inducible IL-2 receptor. Both the production of IL-2 and the expression of IL-2 receptors are pivotal events in the full expression of a human immune response. While the antigen confers specificity for a given immune response, the interaction of IL-2 and the multichain IL-2 receptor determines its magnitude and duration (Leonard *et al.*, 1982; Smith, 1984; Waldmann, 1986; Tsudo *et al.*, 1986).

B. T Cell Receptor Gene Organization

In 1984, using a molecular biological approach to study the receptor, two groups isolated cDNA clones that coded for the β chain of the T cell receptor. They used subtractive hybridization and differential screening to identify a number of cDNA clones that appeared only in T cells but not B cells (Hedrick *et al.*, 1984a,b; Yanagi *et al.*, 1984). A small proportion of these clones were shown to identify genes that rearrange in T cell lymphomas, T cell hybridomas, and clones of functional T cells (Hedrick *et al.*, 1984a; Yanagi *et al.*, 1984). Utilizing this approach, three gene families have been identified that rearrange during the somatic development of T cells (Caccia *et al.*, 1984; Chien *et al.*, 1984a,b; Hannum *et al.*, 1984; Hedrick *et al.*, 1984a,b; Saito *et al.*, 1984a,b; Sims *et al.*, 1984; Siu *et al.*, 1984a,b; Toyonaga *et al.*, 1984; Yanagi *et al.*, 1984, 1985; Hayday *et al.*, 1985; Murre *et al.*, 1985; Yoshikai *et al.*, 1985; Kronenberg *et al.*, 1986) (Fig. 4). Two of these gene families (α and β) encode subunits of the antigen-specific receptor of the T cell. A third gene family, designated the γ gene family, is found to be rearranged in murine and human T cells, although the function of the γ chain has not been defined (Hayday *et al.*, 1985;

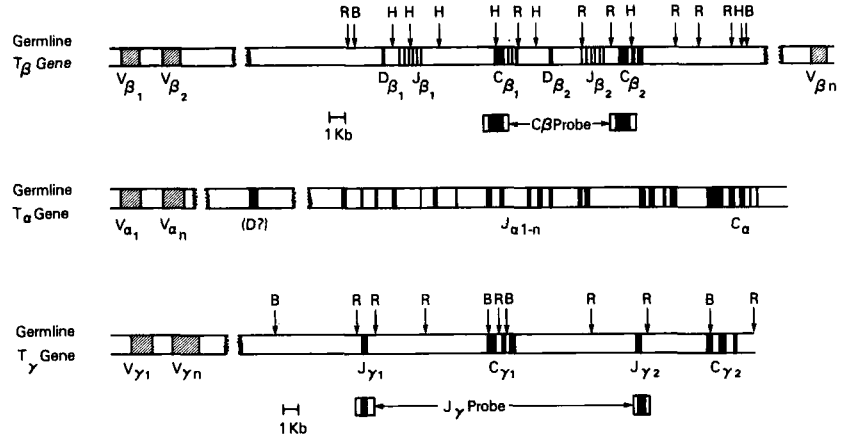

FIG. 4. Schematic diagram of the germ-line arrangement of the T_β, T_α, and T_γ chain genes. The locations of the *Bam*HI, *Eco*RI, and *Hin*dIII restriction endonuclease sites are indicated by the letters B, R, and H, respectively. The T_β gene is composed of multiple variable (V_β) segments and duplicated regions, each including a single D_β, six functional J_β, and a single C_β region. The two C_β genes are present on a single 24-kb *Bam*HI fragment, two *Eco*RI fragments of 4 and 11 kb, and on three *Hin*dIII fragments of 3.5, 6.5, and 8 kb. The germ-line T_α gene utilizes multiple V_α genes, multiple J_α gene segments (>40 J_α segments arrayed over >70 kb), and a single C_α gene made up of multiple exons. The germ-line J_γ gene has duplicated J_γ and C_γ regions. The J_γ probe identifies 1.5- and 3.4-kb J_γ-bearing fragments.

Murre *et al.*, 1985; Quertermous *et al.*, 1986). The primary structure of these proteins was deduced from the DNA sequence of their cDNAs. All three of the T cell receptor gene families (α, β, and γ) have variable, joining, and constant elements that are similar in size and sequence (15–50% homology) to those of immunoglobulins. Each of the chains can be subdivided into 7 regions—a hydrophobic leader region of 18–29 amino acids, a variable (V) segment of 88–98 amino acids, a joining (J) segment of 14–21 amino acids, a sequence of 87–113 amono acid constant (C) region, a connecting peptide, a transmembrane region of approximately 20–24 amino acids, and a small cytoplasmic region of 5–12 amino acids (Kronenberg *et al.*, 1986). The existence of the diversity region element between variable and joining regions has also been established for the β chain molecule. There are many similarities between immunoglobulin and T cell receptor genes in terms of genetic organization, overall structure, signals for rearrangement, and modes of generation of diversity.

The organization of the T_β receptor gene has been most extensively analyzed. These genes are located on chromosome 7 at band q32–35 in humans (Isobe *et al.*, 1985; Morton *et al.*, 1985; Rabbitts *et al.*,

1985a). The human T_β chain gene, in its germ-line form, is composed of discontinuous elements consisting of multiple variable region (V_β) and duplicate sets of diversity ($D_{\beta 1}$, $D_{\beta 2}$), joining $J_{\beta 1}$, $J_{\beta 2}$), and constant ($C_{\beta 1}$, $C_{\beta 2}$) region elements (Fig. 4). The protein deduced from the cDNA sequence shows extensive similarity in its amino acid sequence to the entire length of the variable, joining, and constant regions of immunoglobulin chains. In addition, the relative positions of cystine residues responsible for intrachain disulfide bonds are similar to those of murine and human immunoglobulin molecules. Approximately 50 V_β gene families have been identified in humans (Behlke *et al.*, 1985; Minden and Mak, 1986). Each of the duplicate sets of diversity, joining, and constant region T_β segments contains a single diversity region gene, a joining cluster that contains six functional and one nonfunctional J gene, as well as a single constant region gene. The 12 functional joining elements in the T_β cluster are more than in either the J_H or J_κ clusters in humans. The two highly conserved constant regions are encoded by four domains; the first two encode a single immunoglobulin-like globular domain followed by a shorter domain containing an extra cystine, presumably important in heterodimer formation, and the third encodes a transmembrane region, while the last exon encodes a short cytoplasmic anchor as well as the 3′ untranslated region of the β chain message. The two C_β regions are virtually identical, having only four amino acid differences between them, and do not appear to encode peptides with distinct functions.

The T cell receptor α chain genes have been defined in the mouse and in humans (Fig. 4). In humans the α chain genes are found on chromosome 14 at band 14q11–12 (Caccia *et al.*, 1985; Collins *et al.*, 1985; Croce *et al.*, 1985). There are a large number of T_α variable region genes arranged in families containing a number of members each. Furthermore, there are at least 40 J_α gene segments in a tandem array present in a greater than 70-kb region 5′ to the single C_α gene. The α chain gene cluster in humans contains only one constant region gene that is divided into four exons. Although no D_α gene has as yet been identified, in light of the very large number of V_α and J_α gene segments, the combinatorial diversity available to the T_α cell receptor may substantially exceed that of immunoglobulins.

A third gene family, termed the T_γ gene family, has many properties in common with the α and β genes, including the following: assembly from gene segments resembling the immunoglobulin variable, joining, and constant regions; rearrangement and expression in T cells but not in B cells; a low but distinct sequence homology to immunoglobulin variable, joining, and constant regions; sequences reminiscent of

the transmembrane and intracytoplasmic regions of integral plasma proteins; and a cystine residue at the position expected for a disulfide bond, linking two subunits of a dimeric membrane protein molecule (Hayday *et al.*, 1985). The function of the T_γ gene is as yet unknown. However, recently the T_γ peptide has been shown to be associated with the T3 peptide and an additional peptide termed T_δ in a nondisulfide-linked heterodimer (Bank *et al.*, 1986; Brenner *et al.*, 1986). This γ–δ chain heterodimer comprised of subunits of 40–44 and 55–62 kDa is expressed on a subset of T4/T8-negative and T4-negative T8-positive T cells that do not express a mature message for the T_α and T_β chains, and this novel heterodimer may be a second T cell receptor (Bank *et al.*, 1986; Brenner *et al.*, 1986; Robertson, 1986). In the mouse, the family comprises at least three variable gene segments, three joining gene segments, and three constant gene segments. The human T_γ genes are found on the short arm of chromosome 7 (7p15) (Murre *et al.*, 1985). At least 10 T_γ variable region genes in 4 families are present (Quertermous *et al.*, 1986). There are two joining and constant region segments arranged as J_1-C_1-J_2-C_2 (Fig. 4) (Murre *et al.*, 1985). Essentially all T cells show rearrangements affecting this locus. As with immunoglobulin genes, there appears to be a hierarchy in the rearrangement and expression of T cell receptor genes that have been defined by analysis of transcripts of fetal thymus cells (Royer *et al.*, 1984; Raulet *et al.*, 1985; Samelson *et al.*, 1985). Rearrangements of the T_γ gene occur first. Subsequently, rearrangements of the T_β genes occur, initiating with DJ rearrangements that are expressed in 1.0-kb mRNA. Subsequently, V_β genes are rearranged to this DJ juncture, and 1.3-kb mRNA encoding the complete T_β peptide is transcribed. Finally, T_α genes are expressed, permitting the production and cell surface expression of the α/β heterodimer of the T cell receptor that is expressed in association with the CD3 (T3) peptides in the mature T cell receptor complex.

C. The Recombination of T Cell Receptor Variable Region Genes Generates Diversity

The recombinational signals for the T cell antigen receptor β chain are similar but differ in significant ways from those of immunoglobulin heavy chains (Fig. 3). Sequences similar, but not identical, to the conserved seven- and nine-nucleotide elements flanking immunoglobulins have been observed adjacent to the T cell receptor gene elements. Furthermore, these heptamer and nonamer sequences are separated by nonconserved spacer DNA that may be either 12 or 23 nucleotides long. In immunoglobulins the diversity segment is

flanked by 12-base spacers; thus, according to the 12/23 rule, whereby recombination takes place only between a sequence pair separated by a 12-nucleotide spacer and one with a 23-nucleotide spacer, neither VJ nor DD joining is allowed in immunoglobulins. In the T_β chain, however, the D_β segment is flanked on its 5′ side by an 12-base spacer but on its 3′ side by a 23-base spacer (Fig. 3), thus permitting both VJ and DD joinings (Chien *et al.*, 1984b; Clark *et al.*, 1984; Kavaler *et al.*, 1984; Siu *et al.*, 1984a,b).

Yancopoulos and co-workers (1986) have demonstrated that a common recombinase performs all of the many variable region gene assembly events of B and T cells and that the cell lineage specificity of these events is mediated by regulatory accessibility of the involved gene segments. They introduced DNA constructs with unjoined D_β and J_β gene segments into B cell precursor lines that can carry out immunoglobulin gene rearrangements. In these studies, the T cell segments rearranged as efficiently as immunoglobulin D_H and J_H gene segments. The use of a common recombinase may explain the rearrangement discussed below of T cell receptor genes in B cell and B cell precursor leukemias and of immunoglobulin genes in T cell and T cell precursor leukemias. Other cell lineage-specific features such as chromatin structure may determine which genes rearrange in the two cell types.

The diversification of the T cell receptor β genes employs a number of strategies utilized to generate immunoglobulin diversity (Patten *et al.*, 1984; Behlke *et al.*, 1985; Kronenberg *et al.*, 1986), including multiple germ-line gene segments, combinatorial joining of these gene segments, combinatorial association of polypeptide subunits, and junctional diversity. Both junctional flexibility from variability in the sites at which gene segments may be joined and the random addition of nucleotides to either end of the diversity gene have been demonstrated for the T_β gene. Most groups have not demonstrated somatic hypermutation affecting T cell V_β diversification that would be analogous to the mechanism generating diversity in immunoglobulin genes. T lymphocytes, however, do exhibit somatic diversification mechanisms not found in B cells. Specifically, the D_β gene segment can be linked to the V_β gene segments in all three translational reading frames, whereas in B cells each D_H gene segment is joined in only a single translational reading frame. Finally, as noted above, according to the 12/23 rule that appears to govern recombination, both VJ and DD joints are theoretically possible (Yoshikai *et al.*, 1984; Jones *et al.*, 1985). These alternative rearrangements in the T cell pool might expand the diversity of the T_β gene products.

One question concerning the T cell receptor genes that is being addressed is whether there is a correlation between the different T cell functions (e.g., help, cytotoxicity, or suppression) and the T cell constant region genes expressed. Essentially all helper T cell and cytotoxic T cell lines and hybridomas examined express both α and β genes. Furthermore, there was no evidence for differential use of $C_{\beta 1}$ or $C_{\beta 2}$ genes on the basis of T cell function or MHC class I or II restriction (Kronenberg *et al.*, 1986). The T_γ genes are expressed in thymocytes. In peripheral T cells, there is low-level expression limited to cytotoxic class I-restricted T cells (Saito *et al.*, 1984b; Kranz *et al.*, 1985). The pattern of T cell receptor gene expression is less well defined in suppressor T cells. Twelve of 15 suppressor T cell hybridomas of mice had deleted the β gene locus on both chromosomes derived from the antigen-specific suppressor T cell partner (Hedrick *et al.*, 1985; Kronenberg *et al.*, 1985). Thus, the T suppressor cells do not appear to use the T_β chain in their receptor. The T_α gene has been expressed in the T suppressor hybridomas examined. In contrast, β gene rearrangements were demonstrable in the five human suppressor lines examined. These were not antigen-specific suppressor T cells. Thus, it is possible that certain types of antigen-specific suppressor T cells express a receptor other than the α/β heterodimer. The recently described T3-associated $T_{\gamma\delta}$ heterodimer that may be present on a subset of T cells expressing the T8 antigen is one candidate for such a suppressor cell receptor.

Natural killer (NK) cells kill a variety of target cells without prior immunization or MHC restriction. Certain cloned murine NK cells and human NK lines that express the T3 as well as T11 antigens have T_β gene rearrangements and T cell receptor α transcripts (Ritz *et al.*, 1985). In contrast, human clones with NK activity that express the T11 but not the T3 antigen do not have α transcripts and only produce truncated 1-kb β transcripts that cannot participate in producing a functional receptor (Ritz *et al.*, 1985). Furthermore, rat large granular lymphomas that manifest NK activity retain their β genes in the germline configuration (Reynolds *et al.*, 1985). These results indicate that α/β heterodimer expression is not required for NK activity.

IV. Applications to Clinical Medicine: Immunoglobulin and T Cell Receptor Gene Rearrangements in Human Lymphoid Neoplasms

The analysis of immunoglobulin and T cell receptor gene structure and arrangement has been used in the study of human neoplasms (Korsmeyer and Waldmann, 1984; Sklar, 1985; Waldmann *et al.*,

1985b). The analysis of rearranged immunoglobulin and T cell receptor genes has been of value in defining the origin of neoplasms that were of controversial lineage previously; determining whether abnormal lymphocytic proliferations were monoclonal, oligoclonal, or polyclonal; the diagnosis, defining the extent, and monitoring the therapy of lymphoid malignancies; determining the state of maturation and the causes for failure of maturation of cells of the B cell and T cell precursor series; and obtaining insights into the cause of the malignant transformation of these cells by an analysis of translocations of certain transforming genes to the site of the immunoglobulin or T cell receptor genes in Burkitt's lymphoma, follicular lymphoma, some cases of chronic lymphocytic leukemia (CLL), and adult T cell leukemia (ATL).

Immunoglobulin and T cell receptor gene organization has been analyzed in lymphoid malignancy in an attempt to overcome the limitations of surface marker studies, to provide a sensitive marker for both B cell and T cell lineage and clonality in neoplasms lacking definitive phenotypic markers. This application of molecular genetics is possible because a DNA rearrangement involving immunoglobulin genes is mandatory within cells of B lymphoid lineage and of T cell receptor genes in cells of T lymphoid lineage. Furthermore, these DNA rearrangements result in deletions of the introns containing restriction endonuclease sites during V/D/J joining (Fig. 1). These changes in the location of the restriction endonuclease sites change the size of the restriction fragment bearing the gene being studied, which enables the uniquely rearranged immunoglobulin gene in a monoclonal expansion of B cells to be identified by Southern blot analysis.

To perform such an analysis, high-molecular-weight DNA is extracted from circulating mononuclear cells, lymph nodes, bone marrow, or the skin (Arnold *et al.*, 1983; Sklar, 1985). This DNA is digested with a restriction endonuclease (e.g., *Bam*HI, *Eco*RI, or *Hin*dIII) chosen on the basis of the restriction maps (Figs. 2 and 4) so that it is capable of distinguishing the rearranged from the germ-line immunoglobulin or T cell gene pattern. The digested DNA is size fractionated by agarose gel electrophoresis and is transferred by the Southern procedure (Southern, 1975) to nitrocellulose paper. Hybridization to ^{32}P-labeled DNA probes for immunoglobulins (e.g., J_H, C_κ, C_λ) or T cell receptors (e.g., C_β, J_γ) is performed, followed by washing at appropriate stringency, and autoradiography. Nonlymphoid control DNA may be run simultaneously to identify the germ-line position of the gene in the patient studied. The use of radioactivity as a signal for

detection carries disadvantages related to radiation hazard and the necessity to produce new labeled probes at frequent intervals because of the short half-life of radioactive phosphorus. Nonradioactive methods for labeling DNA probes such as the *in vitro* incorporation of biotinylated deoxynucleotides into DNA fragments are being introduced as alternative probes that can be detected with avidin-linked enzymes or anti-biotin antibodies (Leary *et al.*, 1983).

Schematic presentations of the immunoglobulin heavy and light chain loci and the T cell receptor β, α, and γ loci are shown in Figs. 2 and 4 (Korsmeyer *et al.*, 1983a; Hannum *et al.*, 1984; Murre *et al.*, 1985; Waldmann *et al.*, 1985b; Yoshikai *et al.*, 1985; Kronenberg *et al.*, 1986; Minden and Mak, 1986). The locations of the restriction endonuclease sites for the most commonly used enzymes and DNA probes are outlined in these figures. The germ-line constant κ gene has been found on a 12-kb *Bam*HI fragment. The human λ constant region probe identifies *Eco*RI-generated restriction endonuclease fragments of 14, 8, and 16 kb, in its most contracted form. Multiple allelic forms of the central *Eco*RI fragment exist that are either 8, 13, 18, or 23 kb in size (Taub *et al.*, 1983). To analyze rearrangements involving the heavy chain immunoglobulin gene locus, a J (J_H) probe is most commonly utilized rather than a C_μ probe, since the C_μ gene locus is deleted in a cell with an immunoglobulin rearrangement that has also undergone a class switch from IgM to another isotype. The J_H probe in germ-line tissues is present on a *Bam*HI fragment approximately 17 kb in size. The T_β chain gene has been analyzed using a C_β probe that hybridizes to both the $C_{\beta 1}$ and the $C_{\beta 2}$ gene segments of this gene. In their germ-line form, the gene segments encoding the two C_β genes are present on a 24-kb *Bam*HI fragment, on two *Eco*RI fragments of 4 and 11 kb, and on three *Hin*dIII fragments of 3.5, 6.5, and 8 kb. In occasional cases, an additional 8-kb *Eco*RI fragment may be identified. The T cell receptor J_γ probe hybridizes with both J regions of the T_γ complex present on a 1.5-kb (J_1) and a 3.4-kb (J_2) *Eco*RI fragment in germ-line DNA (Murre *et al.*, 1985; Quertermous *et al.*, 1986). The C region T_γ genes that consist of two closely related gene elements are found on 15-kb (C_1) and 12.5-kb (C_2) *Bam*HI fragments. The large number of J region genes dispersed over a very large segment of DNA in the T_α gene complex makes the accurate assessment of somatic rearrangements of this gene complex difficult at the present time since the restriction enzymes utilized have sites of action between C_α and the more 5′ J_α genes (Yoshikai *et al.*, 1985). That is, a rearrangement of variable region genes to such 5′ J_α region genes does not lead to an alteration in the size of the restriction fragment bearing

the constant α probe. Two approaches are being used to resolve this problem. The first involves the accumulation of probes to each of the J regions that could be used in a fashion analogous to the use of the J-heavy probe for immunoglobulin genes. The other approach is to use enzymes (e.g., *Xho*) with no site of action between a site 3′ to the constant α locus and a site 5′ to all J_α gene elements. The size of the very large restriction fragments generated by such enzymes would then have to be analyzed using special procedures such as pulsed field or periodic inversion field electrophoresis (Schwartz and Cantor, 1984; Carle *et al.*, 1986).

Different patterns are observed that permit one to distinguish clonal B cells from polyclonal B cells and clonal T cells from polyclonal T cell proliferations when digested DNA restriction fragments are analyzed on a Southern blot. A polyclonal population of normal B cells possesses numerous different immunoglobulin gene rearrangements which result in multiple differently sized immunoglobulin-containing DNA fragments after restriction enzyme digestion. When this collection of DNA fragments is analyzed on a Southern blot, no single rearranged band is detected because each rearrangement is below the threshold of sensitivity of this procedure (i.e., <1 to 10% of the cellular DNA analyzed) (Arnold *et al.*, 1983; Cleary *et al.*, 1984a; Minden and Mak, 1986). In contrast, a monoclonal expansion representing a single cell's progeny will have a unique identifying DNA rearrangement pattern specific for that tumor. In this instance, the clonal cells analyzed have a rearranged immunoglobulin gene-containing fragment that is the same size in all cells of the clone. The multiple copies of this unique fragment present in the total DNA from a clonal expansion allow this immunoglobulin gene rearrangement to be detected on a Southern blot as a distinct band different in size from the band representing the germ-line (unrearranged) form of the gene. The detection of clonally rearranged genes by Southern blot analysis has sufficient sensitivity to detect even a minority of clonal cells within tissues admixed with normal cellular elements. In order to estimate the sensitivity of this method, genomic DNA from cells with germ-line immunoglobulin genes was admixed in various ratios with DNA from a monoclonal B cell line bearing specific immunoglobulin gene rearrangements (Arnold *et al.*, 1983; Cleary *et al.*, 1984a). The appropriately rearranged bands were clearly detectable with J_H, C_κ, or C_λ probes when DNA from the monoclonal B cell constitute only from 1 to 10% of the total DNA hybridized. Thus, the presence of a non-germ-line band on a Southern blot is a sensitive and helpful feature for the identification of a clonal B cell population.

All mature clonal B cells invariably display rearrangements of one or both immunoglobulin heavy chain gene clusters and one or more light chain genes (Arnold *et al.*, 1983; Korsmeyer *et al.*, 1983a). In contrast, hematopoietic and lymphoid cells that pursue other than the B cell pathway of development usually retain their immunoglobulin genes in the germ-line configuration (Arnold *et al.*, 1983). In occasional cases, the arrangement of immunoglobulin genes does not fit this pattern. For example, as discussed more extensively below, immunoglobulin heavy chain gene rearrangements have been demonstrated in approximately 10% of leukemic T cell populations and T cell lines examined as well as in certain clonal myeloid cells (Cory *et al.*, 1980a; Arnold *et al.*, 1983; Rovigatti *et al.*, 1984). Furthermore, immunoglobulin mRNA species were also found in some T lymphoma lines, myeloid cell lines, and purified mouse thymocytes. It should be noted that the immunoglobulin heavy chain gene rearrangements that have been demonstrated in T cells have been limited to D/J rearrangements, especially the most 3′ D element and the nearest J segment gene J_{H1}, and have not involved rearrangements of the V_H gene subsegments such as is observed in all mature B cells (Yanagi *et al.*, 1985). Furthermore, all human T cell and myeloid malignancies examined have uniformly retained germ-line (unrearranged) light chain genes (Arnold *et al.*, 1983). Therefore, the detection of both rearranged heavy and light chain genes serves as a B cell lineage-specific marker.

To obtain valid information concerning T cell proliferations, a number of probes and different restriction enzymes should be utilized. The enzyme *Bam*HI, used with a probe to the C_β region, is useful for detecting rearrangements involving either the $C_{\beta 1}$ or $C_{\beta 2}$ regions. The normal germ-line band detected by this combination of probe and restriction enzyme is approximately 24 kb in length. The enzyme *Eco*RI, used in combination with the T cell C_β probe, yields two *Eco*RI fragments of 11 kb ($C_{\beta 1}$) and 4 kb ($C_{\beta 2}$). The $C_{\beta 2}$ gene segment present on the 4-kb *Eco*RI fragment is flanked by *Eco*RI sites with a restriction site between $C_{\beta 2}$ and $J_{\beta 2}$ (Waldmann *et al.*, 1985a). Thus, the size of this *Eco*RI fragment bearing the $C_{\beta 2}$ gene is not altered by any V/D/J rearrangements and the intensity of the band associated with this gene element is identical in all tissues. In contrast, the $C_{\beta 1}$-containing *Eco*RI fragment (11 kb in its germ-line form) is altered in size by most rearrangements affecting V, D_1, or J_1 and is eliminated by rearrangements involving V, D_2, and J_2. It is thus of value to compare the intensities of the 11- and 4-kb bands. In non-T cells these bands are of equal intensity. A decrease in the intensity of the 11-kb band is

observed in polyclonal T cells, indicating that effective or aberrant rearrangements have occurred on both T_β alleles in the majority of such normal cells (Waldmann *et al.*, 1985a). A second mechanism that yields a decreased intensity of the 11-kb band with no detectable non-germ-line band is a clonal rearrangement joining 5′ elements to both J_2C_2 loci leading to deletions of the $C_{\beta 1}$ genes. This pattern can be detected by analyzing the arrangement of the J_2C_2 region of the $C_{\beta 2}$ gene by probing DNA digested with *Hin*dIII. Germ-line DNA digested with *Hin*dIII yields fragments of 3.5, 6.5, and 8.0 kb. The 3.5-kb band contains a $C_{\beta 1}$ region, whereas the 8.0-kb band contains a $C_{\beta 2}$ region. As there is a *Hin*dIII restriction endonuclease site between $J_{\beta 1}$ and $C_{\beta 1}$, rearrangements involving $J_{\beta 1}$ are not detected. However, rearrangements into the J_2 region not detected on *Eco*RI digests are detected on *Hin*dIII digests as a new band on Southern blots. The demonstration of such a non-germ-line band would identify a clonal population with a rearrangement into the J_2 region and distinguish it from a polyclonal T cell population. Utilizing a T cell J_γ probe of the T_γ gene complex, two germ-line *Eco*RI bands of 1.5 and 3.4 kb corresponding to the $J_{\gamma 1}$ and $J_{\gamma 2}$ regions are obtained (Murre *et al.*, 1985; Quertermous *et al.*, 1986). In most clonal T cells, there are rearrangements involving both genes that lead to loss of the bands at the germ-line position and the appearance of two new bands, one for each allele. In polyclonal T cells, the 3.4- and 1.4-kb *Eco*RI bands are retained; however, faint additional bands appear that reflect the limited number of common rearranged bands that are possible due to the small number of variable and joining elements in the T cell γ locus. Such faint bands with this probe should not be misinterpreted as a low proportion of clonal T cells admixed with polyclonal T cells.

In addition to analyzing the arrangement of immunoglobulin genes, the expression of these genes can be assessed using Northern blotting procedures on RNA extracted from lymphocyte populations. This is especially valuable in the study of the T_α gene complex, where, as discussed previously, accurate assessment of somatic rearrangement is difficult due to the large size of the locus bearing J_α genes. Mature T_α mRNA encoding the complete T_α peptide is 1.6 kb in length; however, a smaller 1.3-kb fragment has been described in immature T cells (Yanagi *et al.*, 1985). Similarly, mature T_β mRNA is 1.3 kb in size, whereas an immature 1.0-kb mRNA that does not involve the rearrangement of the V_β gene to the D/J junction can be detected in thymocytes and immature T cells (Yanagi *et al.*, 1985). The mature T cell J_γ mRNA is approximately 1.7 kb in length.

In summary, all mature leukemic T cells examined possess unique rearrangements of both the T_β and T_γ genes, usually affecting both chromosomal loci for each gene that are manifested as a loss of the normal germ-line band and the presence of two new bands demonstrated with each probe on Southern blots. No new bands are demonstrable on polyclonal T cells, but there is a diminution of the 11-kb *Eco*RI band when the C_β probe is utilized. In general, the T cell receptor genes remain in the germ-line configuration in cells other than the T cell series. In occasional cases, as discussed more extensively below, rearrangements of the T_β and T_γ genes have been demonstrated in approximately 10% of mature leukemic B cell populations and at a higher frequency in cells of the B cell precursor lineage.

A. Immunoglobulin and T Cell Receptor Gene Rearrangement Patterns in Mature B and T Cell Malignancies

1. Immunoglobulin Gene Rearrangements in Mature B Cell Malignancy

All malignant cell populations examined from patients with a mature B cell or plasma cell malignancy, such as CLL, Waldenstrom's macroglobulinemia, multiple myeloma, B cell follicular and diffuse lymphoma, B cell prolymphocytic leukemia (PLL), or Burkitt's lymphoma, displayed rearrangements of at least one immunoglobulin heavy plus one light chain gene (Korsmeyer *et al.*, 1983a; Foroni *et al.*, 1984; Melo *et al.*, 1985). Using a J_H probe, in over 80% of cases two bands were observed reflecting an effective V/D/J rearrangement on one chromosome and a presumably nonproductive rearrangement of the immunoglobulin heavy chain genes on the alternative chromosome 14. Individual B cells or clonal B cell malignancies produce only one of two potential types of light chain, κ or λ. Furthermore, each B cell clone uses only a single allele, either the maternally or paternally derived allele of the selected light chain type. Approximately 60% of the time, human B cells express the κ light chain type. The constant (C) region gene is universally present on a 12-kb *Bam*HI fragment in all non-B cells. All κ-expressing B cells demonstrate at least one κ gene rearrangement when compared to this germ-line configuration. The unused or excluded κ gene allele has been found in three different configurations, all of which are nonproductive (Korsmeyer *et al.*, 1983a). The excluded allele may remain in the germ-line form, may be deleted from the cell, or may be rearranged in a nonproductive form (Korsmeyer *et al.*, 1981, 1983a; Korsmeyer and Waldmann, 1984).

Each of these configurations prevents the expression of a particular κ allele and may account for the B cell's capacity to produce only a single κ molecule, the phenomenon of allelic exclusion. The approximately 40% of human B cells that express λ light chains display the obligate λ gene rearrangements. The mechanisms leading to isotypic exclusion, the process by which B cells make one but not both light chain classes, were examined using leukemic clones expressing light chain genes. κ-producing B cells retained their λ genes in the native germ-line configuration, thus accounting for the lack of expression. In contrast, the κ genes within λ-producing B cells were no longer in the germ-line configuration but were either rearranged or deleted (Heiter *et al.*, 1981b; Korsmeyer *et al.*, 1981, 1983a). This finding proved to be true not only of clonal B cell malignancies and continuous λ-producing Epstein–Barr virus (EBV)-induced B cell lines, but was also true of purified λ-producing B cells from the peripheral blood of normal individuals (Korsmeyer *et al.*, 1982). The fact that λ genes appeared to remain in the germ-line configuration in κ-producing B cells, whereas κ genes are rearranged or deleted in λ-producing cells, led to the suggestion of a hierarchy of events involving light chain gene activation in which κ gene rearrangements preceded those of λ. The light chain gene rearrangements initiating with κ would appear to halt when an effective recombination has been accomplished. However, if nonproductive rearrangements of both sets of κ genes have occurred, rearrangements could go on to the λ gene family. Durdik and co-workers (1984), analyzing λ-expressing B cells of mice, and Siminovitch and co-workers (1986), examining λ-expressing B cells in humans, have demonstrated that a uniform deleting element is involved in the loss of κ genes. The sequences 3′ to the deleted element have been the same in all instances examined. A probe to this 3′ region has been used by Siminovitch and co-workers (1986) to show that all losses of κ genes in λ-producing B cells, pre-B cells, and even on the excluded rearranged κ allele in κ-producing B cells, involve this determinant. In 75% of the instances of κ gene loss, this element recombines site specifically with a palindromic signal (CACAGTG) located in the J_κ–C_κ intron. In the remaining 25% of cases of κ gene loss, the element rearranges with upstream sequences, presumably the heptamer signal of V_κ regions. In contrast, the element remains in its germ-line form on all successful κ-producing alleles. It is of interest that the heptamer sequence CACAGTG that is the target for this rearrangement is the same recombinational signal that flanks germ-line V and J regions. This suggests that the same recombinase that activates the κ gene by V/J joining also mediates this deletion. The deleted

region may include genes that produce a transacting factor that inhibits λ gene rearrangements. Thus, the deletion of this segment may be required for λ gene activation. Whatever the mechanism, the results of this work suggest that this element might help ensure isotypic and allelic exclusion of light chains and could play a role in the ordered use of human light chain genes.

Approximately 10% of mature B cell lymphomas manifest clonal rearrangements of their T cell receptor β genes in addition to their heavy and light chain gene rearrangements (Arnold *et al.*, 1983; Pelicci *et al.*, 1985). However, such T cell gene rearrangements do not present problems for lineage assignment, since all of the mature B cell malignancies have a rearrangement of their light chain genes, an event restricted to cells of the B cell/plasma cell series.

Chromosomal translocations represent additional DNA recombinations that occur at the various immunoglobulin gene loci in certain human B cell neoplasms (Rowley, 1980, 1983; Klein, 1981, 1983; Dalla-Favera *et al.*, 1982; Taub *et al.*, 1982; Leder *et al.*, 1983; Croce *et al.*, 1984; Croce and Nowell, 1986). The analysis of such translocations that involve both cellular transforming genes and immunoglobulin genes is providing major insights into the cause of the malignant transformation. With this approach, genes can be identified that are not only involved in the neoplastic process in B cells but also play a role in B cell differentiation and proliferation. A thorough analysis of such translocations is beyond the scope of the present article. Furthermore, the molecular genetics of human B cell neoplasia have been reviewed in Volume 38 of *Advances in Immunology* (Croce and Nowell, 1986). Such translocations will, however, be considered briefly, since they are of value as an aid to the diagnosis of B cell malignancy and aid in monitoring therapy of B cell tumors, and are critical in defining the monoclonal origin of B cell neoplasms that undergo clonal evolution with progressive rearrangements and mutations affecting the immunoglobulin gene loci. One such translocation is characteristically found in Burkitt's lymphoma, a neoplastic condition involving B cells (Dalla-Favera *et al.*, 1982; Taub *et al.*, 1982; Adams *et al.*, 1983; Leder *et al.*, 1983; Croce and Nowell, 1985, 1986; Kelly and Siebenlist, 1985). Three reciprocal translocations have been detected, in each case involving an immunoglobulin gene locus and chromosome 8q24, the site of the c-*myc* oncogene. In 75% of the cases, the cases, the distal end of the long arm of chromosome 8 (q24-qter) translocates to the long arm of chromosome 14 at band q32, the site of the immunoglobulin heavy chain genes (Manolov and Manolova, 1972; Zech *et al.*, 1976). In the remaining 25% of the cases, the

same small segment of chromosome 8 translocates either to the long arm of chromosome 22 at band q11 (16% of cases), the site of the λ light chain genes, or to the short arm of chromosome 2 at band p11 (9% of cases), the site of the κ light chain genes (Lenoir *et al.*, 1982; Emanuel *et al.*, 1984). In the 8;14 translocations, the c-*myc* oncogene is found on the chromosome $14q^+$, while the $8q^-$ chromosome contains the immunoglobulin heavy chain variable region genes. The precise positions of the chromosome breakpoints within the heavy chain locus are not exactly the same from case to case. Burkitt's cells carrying the t(8;22) translocation have $22q^-$ chromosomes that contain the V_λ genes, while the $8q^-$ chromosome contains the C_λ gene. Similar results were obtained by analyzing Burkitt's lymphoma with a t(2;8) translocation, with a breakpoint separating the V_κ and C_κ elements, and a translocation of C_κ to the involved chromosome 8 (Taub *et al.*, 1982; Leder *et al.*, 1983; Croce and Nowell, 1986). In these cases, c-*myc* remains on chromosome 8, and the rearrangement occurs 3′ to c-*myc* in contrast to the 8;14 translocation which occurs 5′. The normal chromosome 14 in the Burkitt's cells contains an effective $V_H/D_H/J_H$ recombination and is responsible for the production of immunoglobulin. Through an analysis of hybrid cells between mouse plasmacytoma cells and Burkitt's lymphoma cells, it has been possible to define the chromosomal source of c-*myc* mRNA. In such cells, only the translocated c-*myc* gene is expressed (Croce *et al.*, 1985). The c-*myc* gene on the normal chromosome 8 is either silent or expressed at extremely low levels in Burkitt's lymphoma cells. Thus, it appears that the uninvolved c-*myc* protooncogene can be regulated normally in response to negative transcriptional control, whereas the involved c-*myc* oncogene fails to respond to these control mechanisms and is expressed constitutively (Leder *et al.*, 1983). This derangement in c-*myc* regulation is a common feature of all cases of Burkitt's lymphomas, in certain acute B cell leukemias that carry the Burkitt-like chromosomal translocations, and in most mouse myelomas where there is a comparable translocation involving c-*myc* gene and the immunoglobulin gene loci (ar-Rushdi *et al.*, 1983; Croce *et al.*, 1983; Erikson *et al.*, 1983; Nishikura *et al.*, 1983). It appears that the constitutive expression of the normal c-*myc* product plays a role in the disordered control of B cell proliferation and contributes to the continuous proliferation of Burkitt's lymphoma cells and thus represents a factor in the pathogenesis of this B cell neoplasm.

Other human lymphomas with a mature B cell phenotype also demonstrate characteristic chromosomal translocations that involve the heavy chain gene locus at chromosome segment 14q32. A transloca-

tion between chromosomes 11q13 and 14q32 is observed in 30–40% of patients with multiple myeloma and in occasional patients with CLL, small lymphocytic lymphoma, and diffuse large cell lymphoma (Nowell *et al.*, 1981; Erikson *et al.*, 1984). In studies of CLL and diffuse B cell lymphomas with the 11;14 translocation, the breakpoints in different tumors were clustered within a small DNA segment, 3.5 kb in length, on band q13 of chromosome 11 (Tsujimoto and Croce, 1984; Tsujimoto *et al.*, 1984a). These authors have inferred that on band q13 of chromosome 11 there is a gene that they have termed bcl-1 (B cell leukemia/lymphoma 1) that is activated by its close proximity to the heavy chain locus. Because a 7 mer–9 mer signal-like sequence with a 12-base-long spacer was identified on the normal chromosome 11 close to the breakpoint, Tsujimoto and Croce (1985) speculate that the t(11;14) chromosome translocation in CLL may be sequence specific and may involve the recombination system for immunoglobulin gene segment (V, D, J) joining. Since the chromosome 11 breakpoints in neoplastic B cells with a t(11;14) translocation cluster within a short segment of DNA, it was possible to use chromosome 11 translocation-specific DNA probes to detect the occurrence of a t(11;14) translocation in malignant lymphoid tissues by Southern blotting analysis (Tsujimoto *et al.*, 1984a).

Chromosome 14q32 is also involved in a reciprocal translocation with chromosome 18 in the majority of patients with follcular lymphoma (Yunis, 1983; Tsujimoto *et al.*, 1984b; Bakhshi *et al.*, 1985). In over 60% of the cases, a 2.8-kb breakpoint region on chromosome segment 18q21 is involved in the t(14;18) translocation in these follicular lymphomas. The break occurs at the 5′ side of the J_H regions of immunoglobulins on chromosome 14q32 where its 5′ flanking sequence retains the immunoglobulin enhancer region in close proximity to the putative transforming gene being introduced. This contrasts with the situation in Burkitt's lymphoma, where the heavy chain enhancer region is usually not retained on the derivative 14 chromosome bearing the c-*myc* oncogene. Thus, the predominant mechanism that alters the transcriptional regulation of the 18q21 element may be different than that controlling c-*myc* in Burkitt's lymphoma. No known oncogene is present on chromosome 18q21; however, a putative transforming gene termed bcl-2 has been cloned from a site near the 18q21 breakpoint. This gene is expressed in a 6.5-kb mRNA, in lymphomas with a t(14;18) translocation, in most pre-B cells, but in much lesser amounts in mature B lymphocytes. Probes have been prepared which identify the 18;14 breakpoint on the derivative (der) 14 ($14q^+$) and der 18 ($18q^-$) chromosome, providing a sensitive and specific molecular

approach to the detection of the t(14;18) translocation in lymphomas. Approximately 60% of unselected follicular lymphomas, 20% of diffuse large cell lymphomas, and 40% of adult undifferentiated non-Burkitt's lymphomas manifested the J_H : 18q21 rearrangements (Bakhshi *et al.*, 1985). Furthermore, in the neoplastic cells of a patient with acute lymphocytic leukemia (ALL) of the B cell type, two reciprocal translocations, one a t(8;14) translocation involving the c-*myc* gene and the immunoglobulin locus and the second a t(14;18) translocation involving the 18q21 transforming gene, have been observed. The translocation of the bcl-2 locus and the c-*myc* locus to the involved immunoglobulin heavy chain loci on two chromosomes 14 may have provided the signals required for the malignant transformation. In certain cases discussed below, T cell lymphomas can rearrange immunoglobulin genes to produce D/J intermediate rearrangements. In two cases of T cell malignancy, Wright and co-workers (1986) demonstrated a translocation that led to a J_H : 18q21 chromosomal juncture. Thus, the same mechanism that results in D/J rearrangements in some T cells may also mediate B-cell-like translocations. Due to the clustering of the rearrangements in a small region on chromosome 18, the 18q21 element should prove useful in detecting translocations in tissues where routine cytogenetics prove difficult or are not available. For example, in biopsy specimens where a difficult differential diagnostic choice occurs between a benign atypical follicular hyperplasia and follicular lymphoma, the detection of this translocation by use of appropriate breakpoint probes would favor a diagnosis of malignancy. In addition, the analysis of the t(14;18) breakpoint region has been shown to be of value in the study of clonal evolution of those follicular lymphomas that are characterized by continuing immunoglobulin gene rearrangements. As discussed more extensively below, follicular lymphomas studied by serial biopsies have been shown to have a conserved t(14;18) breakpoint in each patient, indicating that the neoplastic cells arose from a common clonal progenitor cell (Raffeld *et al.*, 1986). However, in many cases, there were variations in the heavy and light chain gene rearrangements due to secondary rearrangements that represent evolution of subclones within these cells with a common clonal origin. Thus, specific chromosomal rearrangements observed in malignant B cells that involve the immunoglobulin gene locus have provided an opportunity to identify new transforming genes that are involved in the malignant transformation. Furthermore, they have identified genes that may be involved in normal B cell differentiation and/or proliferation. Finally, probes to the breakpoint regions appear to be of value in the diagnosis of some forms of human

B cell malignancy, in defining their clonality, as well as in studying the clonal evolution of such malignant B cell populations.

2. *T Cell Receptor Gene Rearrangements in Mature T Lymphocytic Leukemias*

In normal individuals, circulating mature T cells are a complex mixture of distinct subsets of cells with different and, at times, opposing functions. The study of leukemic T cells that represent an expansion of a single clone of cells that may retain a single immunoregulatory function has been of great value in the analysis of this complex regulatory T cell network. A number of approaches have traditionally been utilized to delineate different human T lymphocyte subsets. The first approach involves the analysis of conventional T lymphocyte markers, such as the classic T cell lineage-restricted marker sheep erythrocyte rosette formation, the definition of the site of tumor involvement (e.g., for T cells, paracortical interfollicular zones of lymph nodes, the paraarteriolar lymph node sheath of the spleen and the skin), and the delineation of a spectrum of cytomorphological characteristics (e.g., cerebriform, convoluted, and multilobate nuclear configurations). In a second approach, heteroantisera or, more recently, monoclonal antibodies to lymphocyte surface antigens are used to define distinct lymphocyte populations. This approach is most valuable when the functional role of the peptide identified by the antibody is known and when it is intimately related to the function of the lymphocyte subset being studied. A third approach involves the use of *in vitro* systems by which antigen-nonspecific helper and suppressor function can be defined (Broder *et al.*, 1976, 1979, 1981; Waldmann and Broder, 1982; Waldmann *et al.*, 1983a). Recently, an additional approach has been added to this list. This approach utilizes the techniques of molecular biology to define the arrangement of T cell receptor and immunoglobulin genes and, thus, to assign a lymphocyte to a particular subset both in terms of cell lineage and state of maturation.

An array of monoclonal antibodies that detect T cell-associated antigens has been described and applied to the study of human leukemic populations (Reinherz and Schlossman, 1980, 1981; Greaves *et al.*, 1981; Haynes, 1981; Greaves, 1982; Knowles *et al.*, 1982, 1985; Talle *et al.*, 1983; Bernard *et al.*, 1984). Nomenclature for the most useful monoclonal antibodies have been standardized by a WHO-IUIS Nomenclature Subcommittee (1984; Bernard *et al.*, 1984) and have been assigned different clusters of differentiation (CD) numbers. Clusters designated 1 through 8 represent human leukocyte differentiation antigens on T lymphocytes, whereas CD25 represents the 55-kDa IL-2

receptor peptide or Tac antigen. CD1 (T6, Leu-6) defines a 49-kDa peptide that is present on 75% of cortical thymocytes and on Langerhans' cells but not on mature T cells. This antigen has been found on some T acute lymphoblastic leukemias and lymphoblastic lymphomas but not on mature T cell malignancies. CD2 (OKT11, Leu-5, 9.6) defines the 50-kDa sheep red blood cell rosette receptor that is present on all T cells that form E rosettes and on most T cell leukemias and T cell non-Hodgkin's lymphomas. CD3 (OKT3, Leu-4, UCHT1) identifies a family of three or more antigens with molecular weights of 19- to 28-kDa that are noncovalently associated with the T cell antigen receptor. This antigen is present on mature thymocytes, approximately 95% of mature peripheral blood T cells, and appears on most cutaneous T cell lymphomas, mature peripheral T cell leukemias, and T cell non-Hodgkin's lymphomas, as well as on a minority of acute T cell lymphoblastic lymphomas. CD4 (OKT4, Leu-3) is a 62,000 M_r antigen present on 75% of thymocytes and mature peripheral T cells. It has been associated with helper T cell function. However, T4 cells also include cells with an array of other functions. T4 cells are involved in interactions that require a recognition of class II molecules (e.g., HLA-DR). In this scheme, T4-positive cells would not only act as helper cells but would also act as cytotoxic or suppressor cells when recognition of class II HLA molecules is involved. The CD4 antigen is found on mature peripheral and cutaneous T cell non-Hodgkin's lymphomas and on mature peripheral T cell leukemias, including the Sézary syndrome and ATL, as well as on rare T cell ALLs and T cell lymphoblastic lymphomas. Recently the CD4 antigen has been identified on certain B cells, monocytes, and brain cells that can be infected with the AIDS-associated virus (HTLV-III/LAV/HIV). CD5 (OKT1, Leu-1, T101) is a 67-kDa antigen present on over 95% of thymocytes and mature peripheral T cells, as well as on less than 5% of mature B lymphocytes. The CD5 antigen has been demonstrated on most T cell malignancies, although in half of the cases of T_γ lymphoproliferative disease the clonal T cells do not bear this antigen. Furthermore, the CD5 antigen can be demonstrated on some sIg-positive B CLL cells and on some B cell non-Hodgkin's lymphomas. CD6 (T12) is a 120-kDa antigen present on mature T cells and on subpopulations of B cells. This antigen has been demonstrated on a few T ALLs, most T cell chronic lymphocytic leukemias (T-CLL), and cutaneous T cell lymphomas, as well as on some B cell CLL cells. CD7 (3A1, Leu-9) is a 40-kDa protein present on over 95% of thymocytes, helper T cells, and on con-A-induced suppressor T cells. It is found on T cell ALL and T cell lymphoblastic lymphomas. In the

majority of cases, mature T cell leukemias, including the Sézary syndrome, ATL, and the T_γ lymphoproliferative syndrome, are CD7 negative. The CD7 antigen is present not only on leukemias of the T cell lineage but is also found on a small percentage of acute myelogenous leukemia cells (Mann *et al.*, 1983; Sutherland *et al.*, 1984). CD8 (OKT8, Leu-2) is a dimer of 30 and 32 kDa present on 75% of thymocytes and on approximately one-third of peripheral mature T cells. It has been reported to be associated with suppressor/cytotoxic T activity. However, it appears, as with the CD4 monoclonals, that the cells defined by CD8 monoclonals differ from others not in terms of their function per se but in terms of the MHC recognition unit with which the T cell interacts. That is, CD8 antibodies appear to be involved in those cellular interactions and functions that involve recognition of class I HLA molecules (e.g., HLA-A or -B molecules). The CD8 antigen has been identified on rare mature peripheral T cell non-Hodgkin's lymphomas and T-CLL cells, as well as on certain T cell acute lymphoblastic leukemias and on T cell lymphoblastic lymphomas. The majority of patients with T_γ lymphoproliferative disease express the CD8 antigen on their circulating cells.

A series of methods have been developed to test for immunoglobulin biosynthesis, and for helper and suppressor activity using *in vitro* lectin-driven immunoglobulin biosynthesis systems, and have been applied to the study of retained immunoregulatory function by mature T cell leukemic cells. These methods and their inherent assumptions and limitations have been reviewed in this series (Waldmann and Broder, 1982). The assays for helper activity take advantage of the observation that pokeweed mitogen is a very T helper cell-dependent stimulator of B cells and that pokeweed mitogen-stimulated B cells in the absence of T cells produce very small amounts of immunoglobulin (Broder *et al.*, 1976). The addition of autologous or allogeneic helper T cells will lead to an enhancement of immunoglobulin production by these rigorously T cell-depleted B cells. Thus, the ability of added leukemic T cells to enhance immunoglobulin production by normal B cells or by the patient's B cells can be used as an index of helper function. Presumably such helper function is dependent on the synthesis and secretion of antigen nonspecific factors required for B cell proliferation and differentiation (e.g., BSF1) by the stimulated malignant T cells. Suppressor cell activity has been assessed by determining the amount of immunoglobulin produced and secreted into the media by pokeweed mitogen-stimulated cocultures of leukemic cells with indicator cells consisting of normal B cells cultured with normal T cells, irradiated T cells, or T8-depleted T cell populations (Wald-

mann and Broder, 1982). The immunoglobulin synthesis in these cocultures is compared to that predicted from the determination of immunoglobulin synthesis by the pokeweed mitogen-stimulated indicator B and T cell population and the leukemic populations when cultured alone.

These functional and phenotypic analyses have been applied to the study of a number of mature T cell leukemias, including the Sézary syndrome and ATL. The cutaneous T cell lymphomas, including mycosis fungoides and the related Sézary syndrome, are malignancies of mature T cells that have cerebriform nuclei. The Sézary syndrome is characterized by exfoliative erythroderma, generalized lymphadenopathy, and circulating pleiomorphic malignant T cells that have the CD2 (T11)-, CD3 (T3, Leu-4)-, CD4 (T4, Leu-3)-, CD5 (T1, Leu-1, T101)-positive CD1 (T6)-, CD7 (3A1, Leu-9)-, CD8 (T8, Leu-2)-negative phenotype. Using an *in vitro* pokeweed mitogen-stimulated immunoglobulin biosynthesis sytem, Sézary syndrome leukemic T cell populations did not synthesize immunoglobulin nor did they function as suppressor cells. However, the neoplastic cells manifested helper activity when cocultured with rigorously T cell-depleted B cells from normal individuals. In the initial report (Broder *et al.*, 1976), the B cells synthesized and secreted from 250 to 5000 ng of IgM when cocultured with the Sézary cells of 6 of the 10 patients studied. In subsequent studies (Waldmann *et al.*, 1984), it was confirmed that Sézary syndrome leukemic T cells not associated with human T cell lymphotrophic virus I (HTLV-I) were dedicated to helper interactions with B lymphocytes.

Uchiyama and colleagues (1977) described a rapidly fatal T cell lymphoproliferative syndrome affecting adults born in the southwest of Japan and referred to this disease as ATL. Like the Sézary syndrome, ATL is a malignant proliferation of mature T cells that have a propensity to infiltrate the skin. Cases of ATL, in contrast to those of the Sézary syndrome, are associated with hypercalcemia, interstitial pulmonary infiltrates, and a very aggressive course in most cases. The ATL cases are clustered within families and geographically, occurring in the southwest of Japan, the Caribbean basin, and in certain areas of Africa. Furthermore, HTLV-I has been shown to be a primary etiological agent in ATL. Approximately 90% of patients with ATL and their spouses have antibodies to HTLV-I. Furthermore, HTLV-I can be demonstrated to be integrated into the genome of the ATL cells by Southern analysis using appropriate probes to this virus. In contrast, patients with Sézary syndrome usually do not have circulating antibodies to this virus. Using the major CD-defined antigens, the ATL

patients have the identical CD2, 3, 4, 5-positive, CD1, 7, 8-negative phenotype as do patients with the Sézary T cell leukemia. Although they share a common phenotype, the ATL cells have a different function when assayed in a pokeweed mitogen-stimulated immunoglobulin biosynthesis system. The ATL cells do not manifest helper activity but rather inhibited immunoglobulin synthesis of pokeweed mitogen-stimulated indicator cells (Uchiyama *et al.*, 1978; Yamada, 1983; Miedema *et al.*, 1984; Waldmann *et al.*, 1984; Morimoto *et al.*, 1985b). Thus, one cannot use the phenotypic expression alone to predict the functional activities of T cell leukemic populations. It has been shown that the generation of an effector of suppression requires an interaction between a radiosensitive CD4-positive inducer cell and a radiosensitive CD8 suppressor precursor cell (Thomas *et al.*, 1981; Morimoto *et al.*, 1985a). Morimoto and co-workers (1985b) suggested that the ATL cells are the leukemic counterpart of the CD4-positive suppressor inducer subset, since in their experience these cells suppress when unirradiated T8 cells are present in the culture but not when the cells are added to a mixture of B cells and T4 cells in the absence of T8 cells. In contrast to these results, other groups have found that ATL cells suppressed immunoglobulin synthesis even when cocultured with an indicator population of B cells and irradiated T cells or of B cells and T cells depleted of T8 cells (Yamada, 1983; Miedema *et al.*, 1984; Waldmann *et al.*, 1984). Furthermore, a long-term T cell line derived from the leukemic cells of one of the ATL patients secreted a 70- to 90-kDa suppressor lymphokine termed SISS-B that inhibited pokeweed mitogen-induced immunoglobulin synthesis by B cells (Greene *et al.*, 1982; Waldmann *et al.*, 1984). These observations are consistent with the view that the HTLV-positive ATL cells function as direct suppressors of B cell maturation and immunoglobulin synthesis. These studies are consistent with the view discussed previously, that T cell populations that react with CD4 and CD8 monoclonal antibodies differ not in terms of their functions per se but in terms of the class of HLA antigen recognized by the T cell. That is, CD8-positive cells appear to be involved in those cellular interactions and functions that involve recognition of class I HLA molecules, whereas T4-positive cells are involved in those interactions that require recognition of class II molecules (Biddison *et al.*, 1982; Krensky *et al.*, 1982; Meuer *et al.*, 1982). In this scheme, different T4-positive leukemic cells could act as cytotoxic or suppressor cells when recognition of class II HLA molecules is involved.

Although the HTLV-I-positive ATL cells could not be distinguished from the HTLV-I-negative Sézary syndrome cells utilizing

antibodies to the CD1 through CD8 antigens, these populations could be distinguished using the CD25 monoclonal antibody (anti-Tac) that identifies the human receptor for IL-2 (Waldmann *et al.*, 1984). Resting normal T cells and most HTLV-I-negative Sézary cells were Tac antigen negative, whereas all HTLV-I-associated ATL cell populations were Tac antigen positive. A molecular analysis of the HTLV-I virus and its protein products suggests a potential mechanism for the universal association between HTLV-I and IL-2 receptor expression. In addition to the presence of typical long terminal repeats (LTRs), *gag, pol,* and *env* genes, retroviral gene sequences common to other groups of retroviruses, HTLV-I and HTLV-II contain an additional genomic region between *env* and LTR referred to as pX (Seiki *et al.*, 1983) or more recently as *tat.* Sodroski and colleagues (1984) demonstrated that this pX or *tat* region encodes a 42-kDa protein now termed the *tat* protein that is essential for viral replication. These authors demonstrated that the *tat* protein acts on a receptor region within the LTRs of HTLV-I and HTLV-II to stimulate transcription. This *tat* protein may also play a central role in directly or indirectly increasing the transcription of host genes such as the IL-2 and the IL-2 receptor genes involved in T cell activation and potentially HTLV-I-mediated T cell leukemogenesis.

The T-CLL has been divided into three subcategories: true T-CLL as well as T cell prolymphocytic PLL and T_γ lymphoproliferative disease, which are considered below (Knowles, 1986). Clinically, T-CLL is characterized by the rapid onset of hepatosplenomegaly, moderate to marked lymphocytosis with bone marrow infiltration, absence of a mediastinal mass, and a tendency to infiltrate the central nervous system, gonads, and the dermis of the skin. The neoplastic cells of T-CLL are generally small to medium in size with a thin rim of anular cytoplasm that contrasts to the abundant azurophilic granular cytoplasm seen in T_γ lymphoproliferative disease. In the majority of cases of T-CLL, the immunophenotypes are identical to those seen with the cutaneous T cell lymphomas. Furthermore, the CD3/CD4-positive, CD8-negative T-CLL cells have been shown to provide helper function for B cell differentiation when analyzed in the *in vitro* pokeweed mitogen-stimulated system (Knowles, 1986). Occasional cases of CD3-positive, CD4-negative, CD8-positive T-CLL have been reported, although in many cases they represent examples of T_γ lymphoproliferative disease.

In 1974, Galton *et al.* (1974) described PLL as an uncommon, particularly aggressive variant of CLL affecting primarily men older than 60 years of age. The clinical features include marked lymphocytosis with

a leukocyte count often greater than 100,000/mm^3, massive splenomegaly, moderate hepatomegaly, and minimal peripheral lymphadenopathy. In the majority of cases, the PLL cells are B cells with surface immunoglobulin. However, T cell PLL has been described usually with a CD3-positive, CD4-positive, CD8-negative phenotype. In occasional cases, the leukemic cells expressed the CD3-positive, CD4-negative, CD8-positive phenotype (Planas *et al.*, 1983). The one leukemic cell population with this phenotype examined manifested helper function.

The arrangement of the T cell receptor β genes has been examined in over 160 cases of mature T cell malignancy. In their germ-line form the gene segments encoding the two C_β genes are present on a single 24-kb *Bam*HI fragment and two *Eco*RI fragments of 4 and 11 kb and three *Hin*dIII fragments of 3.5, 6.5, and 8 kb. Polyclonal T cells have a marked diminution of the intensity of the 11-kb *Eco*RI band when compared with the 4-kb *Eco*RI band defined by the C_β probe; however, there is no new band detectable on Southern blots. In contrast to polyclonal T cells, virtually all malignant expansions of mature T cells examined displayed an identifiable DNA rearrangement when examined with a C_β probe. In virtually all cases the leukemic cells manifested multiple (two or even three) rearrangements with loss of certain germ-like bands as assessed by Southern analysis, supporting the view that most T cells manifest an effective or nonproductive rearrangement of both T_β alleles. Specifically, T cell β chain gene rearrangements were observed in 33 of 33 cases of T-CLL, 52 of 53 cases of cutaneous T cell lymphoma (including patients with the Sézary syndrome or mycosis fungoides), 20 of 20 cases of T cell PLL, 14 of 14 cases studies with ATL, and 39 of 43 cases studied with T cell lymphoma (Toyonaga *et al.*, 1984; Aisenberg *et al.*, 1985; Bertness *et al.*, 1985; Flug *et al.*, 1985; Isaackson *et al.*, 1985; Minden *et al.*, 1985; O'Connor *et al.*, 1985; Pelicci *et al.*, 1985; Waldmann *et al.*, 1985a; Weiss *et al.*, 1985; Minden and Mak, 1986; Rabbitts *et al.*, 1985b).

There was no evidence for an association of any restricted rearrangement pattern with any particular type of neoplasia (Kronenberg *et al.*, 1986). Thus, the demonstration of a C_β gene rearrangement confirms the clonality of these neoplasms but does not provide a tool for differentiating one type of mature T cell malignancy from another. Sixteen mature T cell leukemias have also been examined for γ chain rearrangement (Lefranc and Rabbitts, 1985; Davey *et al.*, 1986; Quertermous *et al.*, 1986). All forms of mature T cell leukemia studied, including five cases of ATL, three cases of Sézary syndrome, five cases

of T-CLL, and three cases of T-cell PLL, manifested T cell receptor γ gene rearrangements. At least five new clonal bands were demonstrable in these leukemias, indicating that there are more T cell γ variable region elements in humans than are observed in mice. In light of the large extent of the genome encoding the J region genes of the T_α gene complex, there are no accurate assessments reported for somatic rearrangements of this complex. Therefore, Davey and co-workers (1986) have assessed the activation of T_α gene complex by looking for T_α mRNA expression. The four mature T cell leukemias (ATL) studied expressed T_β and T_α mRNA, while the rearranged T_γ gene was expressed in the leukemic cells of only one of these four patients. In contrast to the ubiquitous rearrangement of T cell receptor genes in mature T cell leukemias, rearrangments of immunoglobulin genes were very rare. We noted 1 such rearrangement in 10 cases of mature T cell leukemia examined (Waldmann *et al.*, 1985a). Furthermore, only 1 additional J_H rearrangement was observed in 56 additional cases of mature T cell malignancy studied by the groups discussed above who analyzed T_β gene rearrangements as well. In all cases, the immunoglobulin light chain genes remained in the germ-line configuration.

It should be noted that virtually all of the cases of mature T cell malignancy that have been analyzed for T cell receptor gene rearrangement were of the CD3-CD4-positive, CD8-negative phenotype. As noted previously, the T cell receptor has been shown to be an α-β heterodimer in helper and cytotoxic T cells, whereas the receptor on suppressor cells may not utilize the β chain gene. Thus, it would be of great importance to examine mature T cell leukemias with the CD3-positive, CD8-positive, CD4-negative phenotype that function as suppressor cells for T_β, T_α, and T_γ gene expression. It is possible that such cells may utilize a receptor for antigen other than the α-β heterodimer, such as the γ–δ heterodimer.

Specific chromosomal translocations involving the T cell receptor gene loci have been identified in human T cell leukemias that are analogous to the translocations involving immunoglobulin loci in B cell malignancies. In most cases, the translocations in T cell malignancies involve the region of the T cell receptor α gene complex on chromosome 14q11. Four types of translocations involving this locus have been observed. One is a chromosomal inversion involving 14q11 and 14q32 (Hecht *et al.*, 1984; Williams *et al.*, 1984; Zech *et al.*, 1984; Baer *et al.*, 1985; Croce *et al.*, 1985). In the cases examined, this involves a fusion of an immunoglobulin heavy chain variable region element from 14q32 with a T cell receptor joining α gene from 14q11

(Baer *et al.*, 1985; Denny *et al.*, 1986). The V_H–$J_\alpha C_\alpha$ rearrangement was productive at the genomic level and, therefore, may encode a hybrid immunoglobulin/T cell receptor polypeptide. A second form of rearrangement involves a reciprocal translocation involving 8q24 and 14q12 (Erikson *et al.*, 1986; Shima *et al.*, 1986). In these cases the breakpoint had occurred between genes for the variable (V_α) and constant (C_α) regions of the α chain of the T cell receptor. The C_α element had translocated to a region 3′ to the site of the c-*myc* oncogene on chromosome 8 at band q24. In the third form, there was a reciprocal translocation involving 11p13 and 14q12 (Williams *et al.*, 1984; Lewis *et al.*, 1985; Rabbitts *et al.*, 1985a). The 11p13 locus has been implicated in the development of Wilms' tumors. It is possible that either the Wilms' tumor-associated gene or an as yet unidentified gene in this region is involved in the malignant transformation and is altered as a result of its translocation into the T cell receptor α chain locus. Finally, the t(14;18)(q32;q21) translocation that has been associated with B cell follicular lymphoma has been identified in two cases of T cell malignancy (Wright *et al.*, 1986).

B. T and B Cell Lineage-Specific Genes in T and B Cell Precursor Acute Lymphocytic Leukemia (ALL)

The study of ALL has provided important insights into the early events in human lymphoid differentiation (Chessells *et al.*, 1977; Vogler *et al.*, 1978; Reinherz *et al.*, 1980; Broder *et al.*, 1981). Yet considerable controversy has surrounded the cellular origin of such leukemias, as only approximately 20% bear surface markers restricted to T cells and only extremely rare cases possess the cell surface immunoglobulins of a mature B cell (Brouet and Seligmann, 1978). An analysis of immunoglobulin and T cell receptor gene rearrangements in such patients has assisted in defining the lineage of such cells, although it should be emphasized that a proportion of B and T cell precursor leukemias manifest rearrangements of both T cell receptor and immunoglobulin genes. The study of patients with ALL has aided in defining a hierarchy of immunoglobulin as well as T cell receptor gene rearrangements that occur during the maturation of lymphoid precursors and has helped to define those pathogenic mechanisms that lead to disordered lymphocyte maturation.

The arrangement of the elements of the T cell receptor β chain gene has been evaluated in 77 cases of ALL that expressed the CD2 (T11) antigen or formed rosettes with sheep red blood cells. In 76 of the 77 cases studied, a rearrangement of 1 or both of the alleles of the T cell receptor was detected (Aisenberg *et al.*, 1985; Bertness *et al.*, 1985;

Flug *et al.*, 1985; Minden *et al.*, 1985; O'Connor *et al.*, 1985; Pelicci *et al.*, 1985; Rabbitts *et al.*, 1985b; Tawa *et al.*, 1985; Waldmann *et al.*, 1985a; Davey *et al.*, 1986; Minden and Mak, 1986). In those patients with leukemias expressing the T11 antigen analyzed by Davey and co-workers (1986), there were rearrangements of the T_γ gene in all patients as well. Davey and co-workers also examined three ALL cell populations that expressed the CD7 (3A1/Leu-9) but not the CD2 (T11) antigen and were therefore viewed as stem cell or very early pre-T cell leukemias. One of these three cases retained the T_γ and T_β genes in the germ-line configuration, whereas the remaining two cases rearranged T_γ genes alone, retaining their germ-line T_β genes. Nine of the 54 cases that were studied for T cell β gene rearrangement discussed above, as well as 3 of 14 additional cases analyzed by Kitchingman *et al.* (1985), also manifested rearrangements of the immunoglobulin heavy chain but not immunoglobulin light chain genes. This incidence (12 of 67) of immunoglobulin heavy chain gene rearrangements in T cell precursor leukemias is higher than that observed for mature T cell malignancies where the leukemic cells of only 2 of 90 patients examined had rearranged immunoglobulin heavy chain genes.

The large extent of the J region gene locus in the T_α complex makes an accurate assessment of somatic rearrangements at this locus difficult since there are sites for restriction enzyme action between C_α and the more 5′ J elements. Therefore, Davey and co-workers (1986) assessed the activation of the T_α gene complex by examining T_α mRNA expression by Northern analysis and comparing it to T_γ and T_β expression. All mature T cell leukemias examined expressed T_β and T_α, whereas T_γ expression was detected in only one of the four patients studied. None of the pre-T cell leukemia lines expressed T_α; however, all expressed T_β and, with one exception, expressed T_γ as well. Thus, an analysis of T cell receptor gene rearrangements and expression in pre-T cell leukemic cells has led to a better understanding of the events that normally occur in the course of lymphocyte ontogeny. Thus, by examining a spectrum of leukemias of the T cell series that extends from stem cells or very early pre-T cells bearing only the T cell antigen defined by the CD7 monoclonal antibody up to mature T cells, Davey and co-workers provided evidence for a hierarchy of rearrangement of T cell-specific genes. In rare cases, both T_γ and T_β genes remained germ-line; in additional cases, there was a rearrangement of T_γ alone, whereas in the remainder there was a rearrangement of both T_γ and T_β genes. In general, pre-T cell leukemias expressed both T_γ and T_β mRNA, whereas mature T cell leukemias expressed T_β

and T_α mRNA. These studies on human leukemias of T cell lineage parallel the observations of a hierarchy of T cell receptor gene activation in thymocytes of fetal mice and support the view that the T_γ gene locus is rearranged first followed by the T_β gene locus, which is in turn followed by T_α gene activation. Accompanying this hierarchy of gene activation is a coordinate sequence of cell surface antigen expression (Fig. 5) (Davey *et al.*, 1986). The earliest recognizable T cell precursors that rearrange only the T_γ gene express the CD7 antigen alone. Cells subsequently rearrange the T_β gene and begin to express the antigen identified by the CD2 monoclonal antibody. The mature T cell leukemias express CD3, CD4, or CD8 peptides, no longer express C7, and begin to express T_α gene mRNA, and for the leukemias studied no longer express measured quantities of T_γ message. These correlations of cell surface antigen expression and T cell receptor gene rearrangements provide further evidence that T lymphocytic leukemias reflect cells at distinct stages of T cell development (Fig. 5).

One of the first applications of the molecular analysis of immunoglobulin gene arrangement to the solution of important clinical problems was the study of the lineage and state of maturation of neoplastic ALL cells that lack conventional markers of either T or B cells, the then so-called null cell leukemia, by Korsmeyer and co-workers (1983a). These cells of the non-T, non-B form of ALL lack surface immunoglobulins and fail to rosette with sheep red blood cells. All 25 cases in this study, wherein the leukemic cells were not identified by T cell

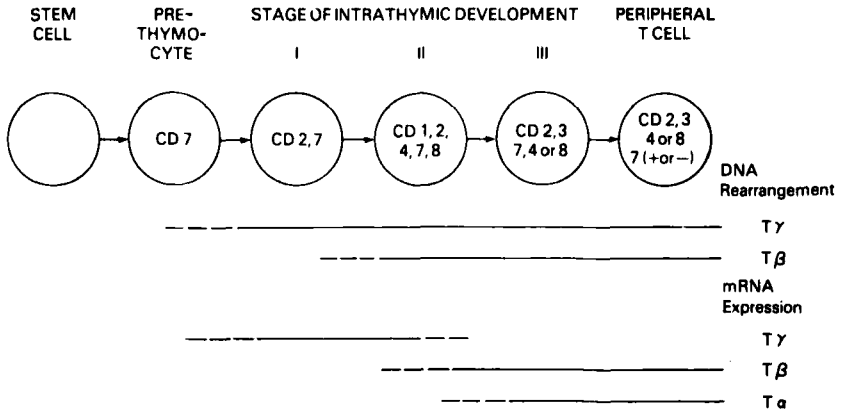

FIG. 5. Coordinate sequence of T cell receptor gene rearrangements paralleled by cell surface antigen expression. T_γ rearrangements and expression precede those of T_β which, in turn, are followed by the expression of the T_α gene locus. This hierarchy of T cell receptor gene expression is paralleled by the acquisition of cell surface antigens in T cell precursor ALLs.

lineage-specific monoclonal antibodies, manifested rearrangements of their immunoglobulin heavy chain genes (Korsmeyer *et al.*, 1986). Our observations have been confirmed by others (Aisenberg *et al.*, 1985; Foa *et al.*, 1985; Minden *et al.*, 1985; Pelicci *et al.*, 1985; Davey *et al.*, 1986; Minden and Mak, 1986), who demonstrated that essentially all (79 of 80 cases) such patients with non-T, non-B ALL have J_H rearrangements. Such rearrangements can be either complete V/D/J junctures or intermediate D/J forms still missing a V_H segment. In 11 of the original 25 cases, these leukemias had progressed to light chain gene rearrangements (Korsmeyer *et al.*, 1983a). These rearrangements, in accord with observations on more mature B cell leukemic light chain gene rearrangements, follow the pattern of recombination predicted by the model in which the κ light chain gene rearrangements precede λ gene rearrangements. Specifically, a series of intermediate light chain rearrangements was observed, in which certain patients had aberrantly rearranged or deleted κ gene elements while retaining there λ genes in the germ-line configuration. Thus, the immunoglobulin gene patterns in these B cell precursor leukemias demonstrated a hierarchy of rearrangements in which heavy chain gene rearrangements preceded light and κ light chain gene rearrangements preceded λ. A coordinate sequence of cell surface antigen expression appeared in synchrony with this developmental cascade of immunoglobulin gene rearrangements (Korsmeyer *et al.*, 1983a; Nadler *et al.*, 1984; Fig. 6). The earliest identifiable B cell precursors displayed heavy chain gene rearrangements and HLA-DR, as well as B4 antigen expression. Later in development, B cell precursors expressed the common acute lymphoblastic leukemia antigen (CALLA) and the subsequently rearranged κ or λ light chain genes. The most mature B cell precursor leukemias frequently expressed the B cell-restricted B1 antigen as well.

Leukemias of immature B cell precursor lymphocytes showed a great tendency to rearrange both immunoglobulin and T cell receptor genes. Nineteen of 63 cases that have been studied manifested such dual rearrangements (Aisenberg *et al.*, 1985; Minden *et al.*, 1985; Pelicci *et al.*, 1985; Tawa *et al.*, 1985; Davey *et al.*, 1986; Minden and Mak, 1986). Three of the five cases of B cell precursor ALL studied by Davey and co-workers (1986) manifested both T_γ and T_β gene rearrangements. It is of note that there is a much higher incidence of T cell receptor gene rearrangements in B cell precursor ALL than in leukemias of mature B cells. Since bands reflecting rearrangements are presumably not lost as B cells mature, the immature leukemias may present patterns that are not on the common pathway of normal B

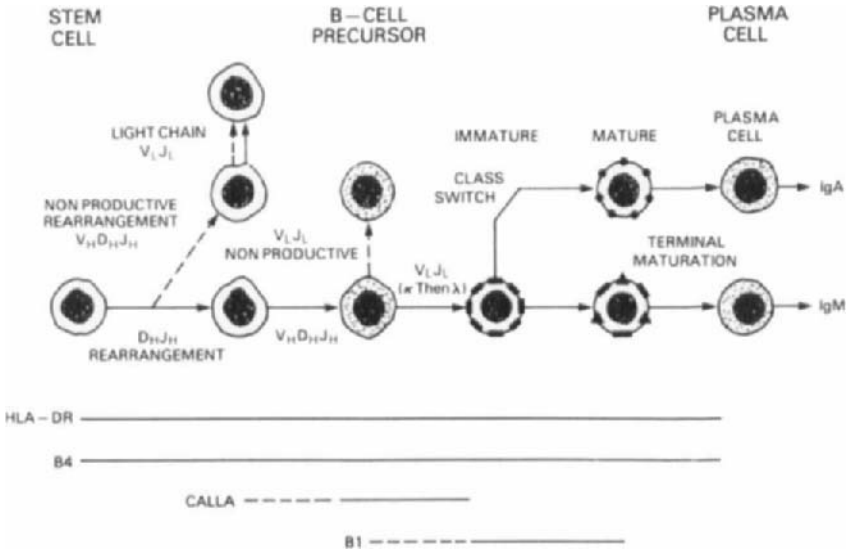

FIG. 6. B cell precursor leukemias represent stages in which rearrangement of the heavy chain D_H segment to J_H segment is followed by the addition of a V_H region. Subsequently, light chains rearrange with κ rearrangements preceding λ. Dashed arrows indicate cells possessing nonproductive aberrantly rearranged genes that may lead to cells lacking the required gene segments for maturation into a cell that can express a cell surface or secreted immunoglobulin molecule. The coordinate sequence of immunoglobulin gene rearrangements is paralleled by the acquisition of cell surface antigens in B cell precursor acute lymphocyte leukemias. Both HLA-DR and the B_4 antigen precede the common acute lymphoblastic leukemia antigen (CALLA) and subsequent light chain gene rearrangement during differentiation. The B_1 antigen appears to have a variable time of onset.

cell maturation. One potential explanation for the high incidence of T cell receptor rearrangement in B cell precursor leukemias is that the leukemic transformation event might have occurred in early precursor cells prior to commitment to the B or T cell lineage. A more likely explanation for this high incidence of T_γ and T_β gene rearrangements in B cell precursor leukemias is that these leukemic cells lack the signals present in more mature cells that terminate immunoglobulin and T cell receptor gene rearrangements. In this view, activation of both B and T cell receptor genes within the same cell may be explained in part by the observation that a common recombinase appears to perform all of the many variable region assembly events of B and T cells (Yancopoulos *et al.*, 1986). Such recombinases responsible for immunoglobulin and T cell receptor gene rearrangements may remain active until an effective stop signal (e.g., mature immunoglo-

bulin heavy chain or mature T cell receptor expression) is produced. Productive V/D/J rearrangements leading to synthesis of a heavy chain polypeptide have been shown to inhibit further heavy chain gene rearrangements (Alt and Baltimore, 1982; Weaver *et al.*, 1985). Thus, if a B cell precursor has a productive heavy chain mRNA produced as a result of an early recombinational event, recombinases may be suppressed, open chromatin sites closed, and no further rearrangements (e.g., of T cell receptor genes) can occur. A transforming event after this stage would lead to an unambiguous B cell malignancy. However, B and T cell precursor leukemias do not have productive immunoglobulin or T cell receptor gene rearrangements and thus lack the signals for terminating recombinase activity. The active common recombinase could continue to rearrange the genes specific for the immune system, including those encoding the genes of the inappropriate lineage. Thus, a transforming event at this stage could lead to the clonal expansion of a population of cells with dual rearrangements.

Although all 25 of the original cases of non-T cell lymphoblastic leukemia studied manifested at least rearrangements of heavy chain genes and in 11 cases light chain genes as well, only 5 cases produced cytoplasmic μ chains and, in only a single case, produced cytoplasmic light chain—in this case a λ light chain. It appears that the previous requirement for cytoplasmic μ chain expression is too stringent a criterion for assigning a cell to the B cell precursor series. Rather, all the non-T, non-B leukemic lymphocyte populations that do not have cells reactive with monoclonal antibodies that define T cells appear to be monoclonal expansions of B cell precursors. A variety of different molecular explanations may account for the frequent lack of immunoglobulin protein production. A small proportion of these leukemias possess an effective rearrangement assembling $V_H/D_H/J_H$ yet do not produce μ chains. Some of these cells can be induced to differentiate with such agents as phorbol esters; when they mature, they produce cytoplasmic and occasionally surface immunoglobulin (Cossman *et al.*, 1982; Nadler *et al.*, 1982). The majority, however, fail to make immunoglobulin following exposure to differentiating agents (Sacchi *et al.*, 1984). Many of these cells possess only incomplete or aberrant rearranged immunoglobulin genes. Cells which possess only intermediate (D_H/J_H) rearrangements may have made a genetic commitment to B cell maturation but are incapable of making intact heavy chains. In other cases, there may have been ineffective or aberrant V/D/J rearrangements for both immunoglobulin heavy chain alleles. In these cases, there may be a set of cells trapped in the B cell precursor

series because ineffective rearrangements of both alleles encoding the heavy chain genes have eliminated the necessary germ-line genetic elements required for the assembly of an effective heavy chain gene. Such cells may have deleted all available germ-line (D_H) gene elements, as well as the heptamer, nonamer, and spacer DNA signal sequences required for rearrangement, and as a consequence may be incapable of forming a complete, activated heavy chain gene. Thus, the somatic recombination joining gene segments not only provides a system to generate antibody diversity but is also error prone, providing a molecular explanation for some of the cell wastage that accompanies differentiation. In summary, ALL cells, nonreactive with monoclonal antibodies identifying T cells, manifest immunoglobulin gene rearrangements that place these cells within the B cell precursor series. Furthermore, the gene patterns observed in these leukemias reveal a cascade of rearrangements in humans in which heavy chain gene rearrangements precede light chain, and κ gene rearrangements precede λ.

C. Immunoglobulin and T Cell Receptor Gene Arrangements Serve as Lineage Markers

1. Lymphoid Blast Crisis of Chronic Myelogenous Leukemia

The molecular genetic analysis with immunoglobulin and T cell receptor gene probes has proved to be of value in determining the cellular lineage of a variety of lymphoid malignancies of controversial origin, including the lymphoid blast crisis of chronic myelogenous leukemias. The cytogenetic and isoenzyme studies have established that chronic myelogenous leukemia is a clonal proliferation arising from a remarkably pluripotent cell (Barr and Fialkow, 1973; Fialkow *et al.*, 1977). Studies of glucose-6 phosphate dehydrogenase isoenzymes in female heterozygotes with chronic myelogenous leukemia revealed that involved granulocytes, erythrocytes, platelets, monocytes, and some lymphocytes displayed only a single glucose-6-phosphate dehydrogenase allele. This finding indicated that, in this disease, cells in multiple hematopoietic lineages could be of clonal origin. Because of this multipotential capacity of the clonally derived cell in chronic myelogenous leukemia, the exact cellular origin and the state of differentiation of cells comprising the blast crisis phases of the disorder have remained uncertain. Occasionally, lymphoblasts from the acute phase have been classified as pre-B cells because of the presence of cytoplasmic immunoglobulin (Minowada *et al.*, 1978; Vogler *et al.*, 1979). However, most cases of lymphoid blast crisis lack

both detectable cytoplasmic and surface immunoglobulin as well as definitive T cell antigens that would allow their clear assignment to either B or T cell lineage. The granulocytic malignant cells in myeloid blast crises from such patients did not show rearrangements of immunoglobulin genes (Bakhshi *et al.*, 1983); however, Bakhshi and co-workers (1983) and Ford and co-workers (1983) demonstrated rearrangements of heavy chain genes in virtually all lymphoid blast crises studied, together with light chain rearrangements in some cases. In contrast, Chan and co-workers (1986) reported a case of a lymphoid blast crisis of Ph^1-positive chronic granulomatous leukemia in which the blast cells had an immature T cell phenotype, germ-line heavy chain immunoglobulin genes, clonal rearrangement and expression of the T cell receptor β gene, and a rearrangement of the breakpoint cluster region (bcr) on chromosome 22. The observations taken as a whole provide evidence suggesting that in the majority of cases lymphoid blast crisis cells are genetically committed B cell precursors, but that rare blast crisis cells may be committed T cell lineage precursors.

2. *Hairy Cell Leukemia*

The malignant cells in patients with hairy cell leukemia also have been of controversial lineage. Evidence favoring a monocytic or histiocytic lineage include the observation that hairy cells often show weak phagocytic activity as assessed by the ingestion of small particles or microorganisms (Braylan *et al.*, 1978; Golomb *et al.*, 1978). Furthermore, these cells also frequently possess tartrate-resistant acid phosphatase, receptors for the Fc portion of IgG, and a roughening of the cell surface on scanning electron microscopy, features occasionally seen with normal monocytes (Jaffe *et al.*, 1974; King *et al.*, 1975; Braylan *et al.*, 1978; Scheinberg *et al.*, 1978). Others have noted T cell-associated antigens on such cells, especially following activation with the lectin phytohemagglutinin (Guglielmi *et al.*, 1980). Other evidence strongly suggests that hairy cell leukemia most often arises from B lymphocytes, since the cells have B cell-associated surface antigens and bear surface immunoglobulins (Jansen *et al.*, 1982a,b; Posnett *et al.*, 1982). The presence of avid Fc fragment receptors (Jaffe *et al.*, 1974) and reports of multiple immunoglobulin isotypes (Jansen *et al.*, 1982a) have raised questions as to whether the immunoglobulin in all cases was actually synthesized by the neoplastic cells. To resolve this uncertainty, the arrangement of immunoglobulin genes was examined by Korsmeyer and co-workers (1983b). All cases examined had rearrangement patterns of at least one heavy and one light chain

gene, characteristic of a mature B cell stage of differentiation. In addition, appropriately sized mRNA from these rearranged genes was detectable and responsible for the surface immunoglobulin. These observations were confirmed by Cleary and co-workers (1984c) and by Meyers and co-workers (1984), who also demonstrated rearrangements of a heavy chain gene and at least one light chain gene in each hairy cell leukemia examined. Thus, in all 35 cases examined, hairy cell leukemia appears to represent a mature B cell malignancy.

3. Diffuse Large Cell Lymphoma

Most non-Hodgkin's lymphomas have been shown to be neoplasms of B cell origin by the demonstration of surface or cytoplasmic immunoglobulin and immunoglobulin gene rearrangements. A minority of non-Hodgkin's lymphomas are considered to be neoplasms of T lymphocytes based on the presence of receptors for sheep erythrocytes and T cell antigens on surface membranes detected with monoclonal antibodies. In addition, a significant percentage of non-Hodgkin's lymphomas, especially in the category of diffuse large cell lymphoma, cannot be classified by the use of monoclonal antibodies. Cleary and co-workers (1985b) analyzed the immunoglobulin and β T cell receptor gene arrangements in 34 diffuse large cell lymphomas that failed to express imunoglobulins or T cell antigens. Twenty-eight cases had both heavy and light chain immunoglobulin gene rearrangements, 2 cases had only heavy chain gene rearrangements, 3 cases had only light chain gene rearrangements, and only 1 case failed to show rearrangements for any of the immunoglobulin genes. None of the cases showed rearrangements of the T cell receptor β gene. These results indicate that the vast majority of diffuse large cell lymphomas that lack definitive B or T cell phenotypic markers are B cell in origin. The reason for the failure of immunoglobulin synthesis by these neoplastic B cells has not been defined. Siminovitch and co-workers (1985) demonstrated immunoglobulin heavy and light chain gene rearrangements in cell lines from seven of eight patients with diffuse histiocytic lymphoma, placing these tumors within the B cell lineage. In one of the cases studied extensively, truncated RNAs for both the heavy and light chain immunoglobulins were shown to be responsible for the lack of surface immunoglobulin.

4. Malignant Histiocytosis Complicating Celiac Disease

Malignant lymphoma complicating celiac disease has been termed malignant "histiocytosis." The view that this tumor was of histiocytic origin was initially founded on morphological grounds, on the pres-

ence of lysozyme (Isaackson and Wright, 1978), on α-1-antitrypsin in the malignant cells, and on enzyme histochemical reactions felt to be characteristic of histiocytes (Isaackson *et al.*, 1982). Such features are not definitive since spurious staining of malignant cells for lysozyme is known to occur due to the uptake of the enzyme from tissue fluids. Furthermore, α-1-antitrypsin has been demonstrated in certain T cell lymphomas and can no longer be considered a specific marker for histiocytes. Isaackson and co-workers (1985) had performed a molecular analysis of the tumor malignant lymphoma complicating celiac disease in four patients. In each case the tumor consisted of large cells with complex nuclei, often with prominent nucleoli and abundant cytoplasm. Each of the tumors reacted with the CD7 monoclonal 3A1, suggesting a T cell origin of these tumors. This was confirmed in three of the four cases by the demonstration of a rearranged T cell receptor β gene; no immunoglobulin gene rearrangements were observed. Thus, the lymphoma complicating celiac disease appears in many cases to be of T cell rather than histiocytic origin.

5. *Acute Lymphocytic Leukemia with a 4;11 Chromosomal Translocation*

Studies of acute leukemias associated with a 4;11 chromosomal translocation have yielded conflicting results when analyzed using monoclonal antibodies with specificities for lymphoid or myelomonocytic antigens. This acute leukemia has morphological, cytochemical, ultrastructural, and immunological features consistent with early myelomonocytic or lymphoid lineage (Parkin *et al.*, 1982). The blast cells from affected patients do not express surface or cytoplasmic immunoglobulin, do not form rosettes with sheep erythrocytes, and do not bear CALLA. Crist and co-workers (1986) demonstrated rearrangement of both allelic heavy chain genes but a germ-line configuration of light chain genes in two cases of acute leukemia associated with the 4;11 chromosome translocation. In addition, Strong and coworkers (1985) demonstrated rearrangement of both heavy chain and κ light chain genes in a cell line derived from a patient with acute leukemia and a t(4;11)(q21;q23) translocation; however, treatment of this line with 12-*O*-tetradecanoylphorbol-13-acetate induced a monocyte-like phenotype demonstrated by cytochemical, functional, immunologic, and electron microscopic studies. On the basis of the phenotypic analysis and immunoglobulin gene rearrangements, the cell of origin may be an early B cell progenitor that shares certain surface antigens with myeloid cells or a stem cell with a potential for both lymphoid and myelomonocytic differentiation.

6. *Hodgkin's Disease, Lennert's Lymphoma, and Angioimmunoblastic Lymphadenopathy*

The nature of the neoplastic cell population in Hodgkin's disease has remained uncertain, largely because the malignant cells are a small component of the involved tissues and have been difficult to purify. It has been suggested that one form of the disease (lymphocyte predominant nodular) is derived from the germinal center, whereas nodular sclerosis is said to arise in the T cell region of the node (Poppema, 1982). A faint but noticeable rearranged T cell receptor β chain arranged band was observed in 3 of 11 Hodgkin's disease lymph nodes studied by Minden and Mak (1986). It is not known whether the faint rearranged bands that reflect only a small proportion of the cells in the lymph node represent the malignant cell or, alternately, a non-malignant clonal proliferation in response to some antigen. No rearrangements of immunoglobulin genes was observed by this group. However, Linch and co-workers (1985) reported a case of lymphocyte-depleted, nodular sclerosing Hodgkin's disease with a terminal leukemic phase where the circulating cells, viewed as Hodgkin's cells, were shown to be of B cell origin by immunological phenotyping and by the demonstration of clonal heavy and light chain immunoglobulin gene rearrangements. Further patients will have to be studied to define whether Hodgkin's disease is a unified entity and to determine the origin of the neoplastic cells. Minden and Mak (1986) also investigated the lineage of so-called Lennert's lymphoma, a heterogeneous lymphoma with a multifocal epithelioid histiocytic reaction. In five of the five cases studied, there was a rearrangement of the T cell receptor β gene but no rearrangement of immunoglobulin genes. In addition, these authors studied patients with angioimmunoblastic lymphadenopathy, a lymphoproliferative disorder that may either recede or progress to a truly malignant form. Nine of 11 cases with angioimmunoblastic lymphadenopathy examined manifested T cell receptor β rearrangements. In addition, four of these nine cases showed rearrangements of immunoglobulin heavy chain genes. One of the 11 patients also showed rearrangement of the κ light chain gene.

7. *Pseudo T Cell Lymphoma*

The molecular approach has been crucial in classifying the cellular origin of the malignant cells in lymphomas of mixed cellularity. Here, diagnostic dilemmas may arise in lymphomas, owing to nondiscriminatory surface marker studies. Arnold and co-workers (1983) examined three cases of diffuse or mixed lymphomas showing a numerical

predominance of T cells (73–89%) (Jaffe *et al.*, 1985), although some B cells were present as well. Furthermore, surface immunoglobulin analysis showed no predominant heavy or light chains present on the minority B cell population. Such cases might easily be classified as T cell lymphomas. However, DNA analysis of samples of the three nodes showed clonal immunoglobulin heavy chain gene rearrangements in each case and, in two, rearrangements of both heavy and light chain loci (Arnold *et al.*, 1983). Furthermore, the T cell β chain receptor genes were in the germ-line configuration (Korsmeyer *et al.*, 1986). Thus, these cases appear to represent monoclonal B cell malignancies with an admixture of large numbers of reactive infiltrating polyclonal T cells.

D. Definition of Clonality Based on Immunoglobulin and T Cell Receptor Gene Rearrangements

One of the most important applications of the analysis of immunoglobulin and T cell receptor gene rearrangements involves the distinction between polyclonal, oligoclonal, and monoclonal lymphoproliferative disorders. Attempts to determine if an individual lymphoid proliferation is monoclonal have classically used three standard approaches—the demonstration of a specific cytogenetic abnormality (Rowley and Fukuhara, 1980), the expression of only a single glucose-6-phosphate dehydrogenase allele within the tumor of a heterozygous female (Fialkow *et al.*, 1973), or the determination that only a single immunoglobulin light chain, either κ or λ is expressed (Levy *et al.*, 1977; Zulman *et al.*, 1978). Each of these approaches has limitations. The glucose-6-phosphate dehydrogenase studies are not applicable to males or to the majority of females that are homozygous at this allele. Although the determination of light chain expression has been used more widely, it too has limitations. First, various technical problems such as nonspecific binding of antibody to highly avid Fc receptors may confound the analysis of clonality based on the restriction of surface immunoglobulin light chain type. Furthermore, lymphoid tumors frequently contain an admixture of cell types with the malignant clone constituting only a small minority, leading to ambiguous results. Furthermore, the lack of detectable surface immunoglobulin within the B cell precursor malignancies limits the evaluation in this large group. Finally, there are no universally acceptable cell surface markers available to define clonality for T cells.

To overcome these limitations, immunoglobulin and T cell receptor gene analysis in lymphoid neoplasms have been used as a sensitive tool to assess clonality. The DNA is extractable from fresh or frozen

specimens, enhancing the applicability of this method for analyzing routinely available clinical material. In addition, it is important to emphasize that the immunoglobulin and T cell receptor gene rearrangement is a marker at the DNA level. Thus, it does not require effective gene rearrangements and the final expression of the antibody or T cell receptor product for its detection. In addition, clonal B or T cells can be detected when admixed with polyclonal cells. That is, a new non-germ-line band on Southern blots probed with the appropriate immunoglobulin or T cell receptor probe can detect a clonal B or T cell population even when its DNA constitutes only 1–5% of the mixture. Finally, by the use of multiple probes (e.g., heavy chain, κ and λ light chain) simultaneously, one can obtain a unique identifying DNA rearrangement pattern specific for a given tumor. A possible difficulty associated with the analysis of immunoglobulin and T cell receptor gene rearrangements in lymphoid proliferations is the identification of apparent rearrangements that are actually due to inherited polymorphisms in immunoglobulin or T cell receptor gene DNA. In practice, such polymorphisms appear to be rare for the heavy chain, κ, and T cell receptor loci studied with the probes and restriction enzymes that are used. As noted above, λ chain polymorphisms do appear that consist of the acquisition or loss of multiples of a 5-kb DNA sequence resulting in a small set of predictable variant bands. An absolute control against such inherited polymorphisms is the parallel analysis of nonlymphoid DNA, for example, that of buffy coat or fibroblasts from the same patient.

The definition of clonality based on immunoglobulin or T cell receptor gene rearrangements has been used in a number of circumstances to address important biological and clinical issues. In the analysis of obvious lymphoid malignancies, gene rearrangements have been used to distinguish truly monoclonal proliferations from single clones which have undergone clonal progression with the development of subclones *in vivo* that have different immunoglobulin gene rearrangements and these, in turn, from truly biclonal or oligoclonal proliferations. This approach has been used to address controversies over whether observed T cell proliferations in certain diseases (e.g., T_γ lymphoproliferative disease) reflect a clonal T cell proliferation or a polyclonal expansion of immunoregulatory cells. The detection of clonal populations of B or T cells has also been an important diagnostic factor that helps to distinguish a malignancy from an atypical lymphoid hyperplasia. Finally, this approach has been of considerable value in monitoring the efficacy of treatment of B and T cell

neoplasia and in the early detection of recurrences of such tumors following therapy.

A number of different patterns have been demonstrated when immunoglobulin and T cell receptor gene rearrangements have been used to define the clonality of lymphoproliferative disorders. In some cases, B cell leukemias and lymphomas have been shown to be monoclonal with an identical immunoglobulin gene rearrangement pattern in all sites within the patient and at different stages of the patient's disease. Moreover, the number of rearranged bands in each case (no more than two rearranged bands for each light chain or heavy chain locus) is consistent with only a single major clone. In other patients, DNA analyses of tissues obtained from different sites or at various times showed different clonal immunoglobulin gene rearrangements. In such cases the individual tumors appear to arise from different B cell clones. In yet other cases, there was evidence of clonal progression. In these latter cases, different lymphoma cells derived from a single malignant transforming event were nevertheless shown to have different immunoglobulin gene rearrangements and, thus, only appeared to be biclonal populations.

One area where this analysis has been particularly helpful has been in the study of lymphoproliferative disorders arising in immunosuppressed or immunodeficient patients receiving organ or bone marrow transplants. There has been considerable speculation about whether such lesions in patients receiving organ transplants should be considered lymphomas or virally induced hyperplasias (Fizzera *et al.*, 1981). In support of this latter view, many of the cells of these proliferations have been shown to harbor EBV (Hanto (1981). To address this issue, Cleary and co-workers (1984b) examined tissue specimens from 10 cases of lymphoproliferative disease that occurred in immunosuppressed recipients of cardiac transplants. Specimens from nine of these patients lacked cellular immunoglobulin; however, analysis of DNA extracted from these tissues identified all specimens as containing clonal rearrangements of immunoglobulin DNA. Thus, the lymphoproliferative disorders in these recipients of cardiac transplants were shown to be clonal neoplasms at an early stage of maturation. In a subsequent study (Cleary and Sklar, 1984), additional tissue was examined from five of the patients of this original study. DNA analysis of tissue obtained from several sites or at various times showed different clonal immunoglobulin gene rearrangements for each of the three immunoglobulin gene loci. Although no individual biopsy specimen produced more than two rearranged bands per immunoglobulin gene

locus, each separate mass contained a distinct clonal population of B cells as judged by variation from biopsy to biopsy. A similar pattern was shown by Shearer and co-workers (1985) in the B cell proliferations observed after bone marrow transplantation of a patient with severe combined immunodeficiency. A number of patients treated with bone marrow transplants for severe combined immunodeficiency have developed B cell proliferations, expecially when histoincompatible T cell-depleted bone marrows were utilized. Shearer and co-workers (1985) studied a 12-year-old boy with severe combined immunodeficiency who had been kept in a gnotobiotic environment since birth who received a T cell-depleted bone marrow transplant from a histoincompatible sibling. The patient developed a systemic illness characterized by fever, thrombocytopenia, gastrointestinal pain and bleeding, and died on day 124 following transplantation. At postmortem examination, the patient had multiple tumor-like B cell proliferations that were recipient in origin in numerous organs. The EBV genome was discovered in all tissues. Two of the patient's lesions examined contained monoclonal but different B cell proliferations as indicated by immunoglobulin gene rearrangements. Five other lesions in this patient corresponded more closely to polyclonal lesions. In these latter cases, multiple faint non-germ-line bands could be demonstrated that could indicate the initial emergency of dominant B cell clones from a polyclonal mass of proliferating B cells. In the two other cases discussed above, this process appeared to have progressed to a more advanced stage in which an overwhelming proportion of cells within each lesion was composed of a single clone of B cells. These findings in this bone marrow transplantation situation provide evidence for the evolution of EBV-induced polyclonal activation of B cells to oligoclonal B cell proliferation and finally to monoclonal B cell lymphoma. Cleary and co-workers (1985a) demonstrated that cotton-top tamarins inoculated with EBV developed multiple large cell lymphomas that contained multiple copies of the EBV genome. Hybridization of tumor DNA with immunoglobulin gene probes showed that each lymphoma was oligo- or monoclonal in origin; yet different tumors from the same animal arose from different B cell clones. Thus, the virus induced multiple transformation events in tamarins *in vivo* to cause malignant tumors analogous to the EBV-associated lymphomas of patients receiving organ and marrow transplants. Truly biclonal tumors have also been demonstrated among B cell lymphomas that show different histologic forms. Van Dongen *et al.* (1984) examined a patient with Richter's syndrome, a syndrome characterized by the development of histiocytic lymphoma or Hodgkin's disease in the

course of CLL. In the patient studied, there were different immunoglobulin light chain and heavy chain gene rearrangements in the CLL cells compared with non-Hodgkin's lymphoma cells of this patient, suggesting independent B cell malignancies in the same host. Siegelman and co-workers (1985) showed that, among B cell lymphomas that manifested different histologic forms (e.g., small versus large cell lymphoma), histologically distinct biopsy specimens contained different clones in three cases on the basis of the demonstration of distinct immunoglobulin gene rearrangement and that different deletions of DNA had occurred in the heavy chain genes between histologically distinct biopsies in two other cases. Only two of the seven cases showed identical rearrangements of all immunoglobulin genes in DNA derived from different sites regardless of the changes in tumor histology.

In yet other cases, apparently biclonal populations did not reflect two independent malignant transformation events but were rather shown to be derived from descendants of a single abnormal cell that underwent clonal evolution to distinct subpopulations of the clone. In such cells showing clonal evolution, a genetic marker was demonstrated in all of the cells that identified them as part of a single clone yet immunoglobulin or T cell rearrangements that differed from one subpopulation of cells to the next were present. In some cases the common genetic element was a karyotypic abnormality, as in the case discussed above studied by Bakhshi and co-workers (1983), where cells from a single patient with chronic myelogenous leukemia obtained during two separate episodes of lymphoid blast crisis showed the same Philadelphia chromosome and 45 XY-7 karyotype and the same rearranged heavy chain genes yet manifested different light chain gene rearrangements, indicating that the two blast crises represented unique subclones at different stages of B cell maturation. The integration of a virus in a particular chromosomal site has also been used as a genetic marker of clonal origin. This approach was used to show the common clonal origin of the HTLV-I-associated adult leukemic cells that manifested different T_β gene rearrangements in a patient studied before and after a remission induced by monoclonal anti-Tac antibody therapy (Waldmann, unpublished). Two additional approaches have been used to show the common clonal origin of cells undergoing clonal progression in patients with follicular B cell lymphomas. In some cases an identical immunoglobulin gene rearrangement for at least one of the rearranging immunoglobulin gene loci common to all of the malignant cells could be demonstrated. In other cases all of the cells were shown to share an identical

t(14;18)(q32;q21) translocation. A number of groups (Sklar *et al.*, 1984; Giardina *et al.*, 1985; Meeker *et al.*, 1985; Raffeld *et al.*, 1985), studying patients with follicular lymphoma, were able to demonstrate tumors apparently composed of two populations of cells because they expressed different immunoglobulin molecules that could be distinguished by monoclonal antibodies directed to the idiotypes expressed on the cell surface immunoglobulins. In some of these cases, the cells from a given patient were shown to have a common clonal origin by manifesting one or more common immunoglobulin gene rearrangements, whereas in others an identical translocation was present. Malignant cells from a given patient separated into idiotype-positive and idiotype-negative cells showed a variety of alterations that led to the differences in the idiotype expressed. In rare cases the different subpopulations showed different heavy chain gene rearrangements identified on Southern analysis or different light chain class rearrangements reflected both by different cell surface light chain expression and different light chain gene rearrangements. More commonly, the two cell populations show different κ gene rearrangements on Southern analysis. Differences due to point mutations, reflecting the hypermutability of immunoglobulin variable regions following their rearrangements, were the most common distinguishing events in the subpopulations. Raffeld and co-workers (1986) showed a similar clonal evolution of B cell follicular lymphomas demonstrated by alterations of immunoglobulin gene rearrangements in cell populations that had an identical chromosomal translocation. In 65 to 80% of follicular B cell lymphomas, a translocation of a chromosomal segment from 18q21 to the site of the immunoglobulin genes on chromosome 14q32 can be identified. This translocation involves a 2.8-kb breakpoint on chromosome segment 18q21 that mediates a translocation to the 5′ side of the J_H region of immunoglobulins. Raffeld and co-workers examined serial biopsies from 16 B cell follicular lymphoma patients. These 16 cases showed variations in immunoglobulin gene patterns over time. Focusing on three patients with J_H : 18q21 rearrangements that remained unchanged throughout 5 to 10 years, these authors demonstrated that all of these cases displayed further rearrangements of the heavy chain genes on chromosome 14 within evolving clones of the original tumor. Furthermore, λ light chain genes underwent rearrangements over time in two cases, yet the κ gene rearrangements remained unchanged. It appears that the variations in heavy and light chain genes over time in these patients were secondary rearrangements reflecting clonal progression occurring at a mature B cell stage of development. The common t(14;18) breakpoint in each patient in-

dicated that the evolving neoplastic subpopulations arose from a common clonal progenitor cell.

The demonstration of clonal progression and of heterogeneity of the idiotype within lymphoma tissue is of considerable biologic interest and is also of critical importance in developing therapeutic protocols involving the use of anti-idiotype therapies for the treatment of lymphoid cancers. Using monoclonal antibodies directed toward the idiotype of B cell lymphomas, Miller and co-workers (1982) obtained a complete prolonged remission in the first patient treated. However, in the subsequent 10 patients, no complete remissions were observed. At least 2 and possibly 4 of the 11 patients in this series had idiotype mutants which would have led to the failure to kill all of the malignant cells (Meeker *et al.*, 1985). The magnitude of this problem has not been fully defined; however, it would appear to be a common problem. It has been estimated that the mutation rate in the whole immunoglobulin variable region of a normal B cell clone responding to an antigen is as high as 10^{-3} per base pair per cell division (Max *et al.*, 1979). Furthermore, Teilland and co-workers (1983) have suggested that a spontaneously altered idiotype would be present in otherwise identical cells in as high as 1 in 100 cells in culture. These observations suggest that, if future attempts at anti-idiotype therapy of B cell malignancy are attempted, it may be appropriate to employ several different anti-idiotype antibodies, each directed at a different idiotope to reduce the probability of tumor escape due to idiotype variation (Meeker *et al.*, 1985). Since T cells do not appear to use hypermutation of the variable region to generate diversity, anti-idiotype therapy of mature T cell malignancies may be more appropriate than such therapy of B cell tumors.

The definition of clonality among cells of the B or T cell series may be of value in the diagnosis of aberrant lymphocytic proliferations. The diagnosis of malignant lymphoma dependent on histologic examination of tissue biopsies is one of the pathologist's most difficult tasks. Although the majority of such biopsies are unambiguous, a significant minority present serious problems since antigenically stimulated polyclonal lymphocytes may resemble neoplastic lymphocytes. The demonstration of clonality of cells within an aberrant B cell proliferation may aid in the diagnosis of malignancy. For example, Arnold and co-workers (1983) have used this approach to demonstrate a clonal B cell component in the lymphoproliferative lesions of a patient with the Wiskott–Aldrich syndrome. Patients with the Wiskott–Aldrich syndrome, a syndrome characterized by thrombocytopenia, eczema, and an immunodeficiency state, have a high incidence (20–50%) of

lymphoid tumors, predominantly of the B cell series (Waldmann *et al.*, 1972). Arnold *et al.* (1983) reported on a 15-year-old immunodeficient boy with the Wiskott–Aldrich syndrome who developed progressive and marked generalized lymphadenopathy. Histologically, the differential diagnosis between follicular lymphoma and atypical follicular hyperplasia was exceedingly difficult since surface marker analysis of a biopsy specimen showed no evidence of a clonal process, and both κ- and λ-bearing B cells as well as many T cells were present. However, DNA from this lymph node contained clonally rearranged immunoglobulin heavy and light chain genes apparently derived from only a small minority of the cells of the node. Clonality is clearly not tantamount to malignancy; for example, benign monoclonal gammopathies and expansions of a B cell clone in response to an antigenic stimulus are examples of clonal processes that are not malignant. However, benign monoclonal gammopathy rarely leads to clinically significant lymphadenopathy. The demonstration of clonality does provide a useful piece of information that aids in diagnosis. Furthermore, serial examination of such patients will reveal whether immunoregulatory controls will prevent further clonal expansion or whether the minority clone represents the early stages of a malignant expansion that will eventually dominate. The distinction between an inflammatory infiltrate of T cells and a monoclonal malignant process can be particularly difficult in T cell infiltrates of the skin and in the draining lymph nodes accompanying such skin infiltrates. Histologic diagnosis of mycosis fungoides may be difficult, especially in lymph nodes that show changes associated with chronic skin disease. In this regard, Weiss and co-workers (1985) demonstrated clonal rearrangements of T cell β chain receptor genes in all cases that contained histologically unambiguous mycosis fungoides. Furthermore, clonal rearrangements also were found in seven of nine lymph nodes removed from such patients with mycosis fungoides that were considered histologically to contain only benign lymphadenopathy. In the one case examined, biopsy tissues obtained from the skin of such a patient showed a T cell receptor β gene rearrangement. This approach may be especially valuable in offering a means of documenting malignant transformation arising in the context of a benign polyclonal proliferative disease. It should be noted that, in many cases, mycosis fungoides develops in skin previously affected for many years by chronic dermatitis.

Immunoglobulin and T cell clonal gene rearrangements can also serve as sensitive tumor-specific markers that have improved the ability to identify persistent tumor following therapy and that facilitate

the early detection of a recurrence. We have utilized clonal T_β gene rearrangements to monitor treatment of patients with ATL (Waldmann *et al.*, 1985c). Since this is a leukemia of mature-appearing T cells, this may be difficult with conventional approaches. One patient treated with anti-Tac monoclonal antibody had a remission of the Tac-expressing leukemic T cell population. During remission, the peripheral T cells no longer showed a rearranged T_β gene fragment that was the hallmark of the clonal expansion of T cells in this patient. Clonal cells with a rearranged band were subsequently demonstrable and anticipated morphologically identifiable malignant cells in the peripheral blood. Wright and co-workers (1985) used a similar analysis of immunoglobulin and T cell receptor β gene rearrangements to define clonal persistence in patients with acute lymphoblastic leukemia. Germ-line gene patterns were observed on bone marrow samples obtained from 9 of 11 patients who were in clinical remission. However, persistent clonal cell populations were demonstrable in aspirates from the two other patients. These two patients had a clinical relapse at 6 and 9 months following the identification of persistent clonal cells in the marrow. Thus, the demonstration of clonal cells in patients in clinical remission without evidence of disease may be of importance in identifying patients with persistent leukemia following therapy.

The analysis of a T cell receptor gene rearrangement has been of value in addressing controversies over whether disorders associated with an expansion of T lymphocyte populations reflect a monoclonal expansion of T cells or a polyclonal expansion of immunoregulatory cells. For example, there has been a controversy concerning the clonality of the CD8 (T8)-expressing cell populations in the syndrome termed T_γ lymphoproliferative disease, T cell lymphocytosis, or chronic T_γ cell leukemia that is characterized by lymphocytosis of large granular lymphocytes and that is associated with granulocytopenia and anemia. The clinical course of these patients is characterized by an increased incidence of infection, mild to moderate splenomegaly, and, in one-third of the patients, long-standing arthropathy, serologically identical to rheumatoid arthritis (Aisenberg *et al.*, 1981; Bagby *et al.*, 1983; Chan *et al.*, 1984; Reynolds and Foon, 1984). The patients have a moderate lymphocytosis with a mean lymphocyte count at presentation of 8000 per cubic millimeter. Most of the patients have associated neutropenia; half of the cases have thrombocytopenia, and a small number of patients have red cell aplasia or hypogammaglobulinemia. The morphologic characteristics and the surface markers of the cells from patients with chronic T_γ lymphoproliferative disease are similar to those of normal large granular lymphocytes. In

the majority of cases, the cells are large cells with a high cytoplasm-to-nucleus ratio that have azurophilic cytoplasmic granules. In the majority of patients reported with T_γ lymphoproliferative disease, the expanded cell population in the blood expressed the CD2 (T11), CD8 (T8) HNK1-positive phenotype. Usually, the cells expressed the CD3 (T3)-positive phenotype, whereas in approximately 50% of the patients studied the cells did not express the pan T cell antigens CD5 (OKT1/Leu-1) and CD7 (Leu-9/3A1). In many of the patients the circulating large T_γ lymphoproliferative cells suppressed T cell mitogenic responses and, in a minority, suppressed immunoglobulin production by cocultured pokeweed mitogen-stimulated T lymphocytes. Nearly all of the cases studied for antibody-dependent cellular cytotoxicity were positive, whereas only one third of the cases studied had NK activity.

The proliferation of large granular lymphocytes in patients with T_γ lymphoproliferative disease is a very indolent process with clinical features dominated by the granulocytopenia. In addition, in some patients spontaneous remissions have been observed. On the basis of these clinical observations, some investigators (Aisenberg *et al.*, 1981; Rümke *et al.*, 1982) have suggested that this disorder may be a reactive rather than a neoplastic process. Other individuals have viewed this as a form of chronic leukemia. In our own studies (Waldmann *et al.*, 1985a; Winton *et al.*, 1986; and unpublished observations), we have demonstrated a clonal rearrangement of the T cell β and γ genes in 7 of 10 patients with T_γ lymphoproliferative disease associated with granulocytopenia. Analogous evidence for a clonal expansion in the majority but not all patients has been reported for about 80% of patients studied by other groups (Aisenberg *et al.*, 1985; Flug *et al.*, 1985; Knowles, 1986). Patients with a proliferation of large granular lymphocytes that express the T11, HNK1, but not the T3 antigen present a different pattern of T cell receptor gene rearrangement and expression. In general, such patients retained their T cell β and γ genes in the germ-line configuration (Chan *et al.*, unpublished observations; Rambaldi *et al.*, 1985). T_γ and T_α mRNA was not expressed in the cultured cells from such patients, but a 1.0-kb truncated T_β transcript could be demonstrated. These observations in the two forms of T_γ lymphoproliferative disease, those that are T3 positive and those that are T3 negative, parallel the observations of Ritz and co-workers (1985), who studied T cell receptor gene arrangement and expression in a series of clones of human NK cells. These authors demonstrated that T11-positive, T3-positive NK clones expressed mature T_α and T_β mRNA transcripts, whereas T11-positive, T3-negative NK clones ex-

pressed only 1.0-kb truncated T_β transcripts without T_α transcripts. Thus, T_γ lymphoproliferative disease appears to be a heterogeneous disorder. In the majority of patients, despite its relatively unaggressive course, this disease is a clonal expansion of large granular lymphocytes expressing the T3, T8, HNK1-positive phenotype.

E. Immunoglobulin Gene Defects in Heavy Chain Disease

Human heavy chain disorders (α chain disease, μ chain disease, and γ chain disease) represent expansions of B lymphoid cells that secrete truncated monoclonal immunoglobulin heavy chains not linked to light chains. The secreted heavy chains have had deletions involving one or more domains. Molecular analyses have been performed on patients with μ and γ heavy chain disease (Alexander *et al.*, 1982; Bakhshi *et al.*, 1984, 1986; Guglielmi *et al.*, 1984). The clinical features of μ chain disease are those of a long-standing CLL with progressive hepatosplenomegaly. Bakhshi and co-workers (1986) have examined the leukemic cells of a patient with a μ heavy chain disease and demonstrated a truncated μ chain of M_r 58,000. The protein started at position 5 of the first domain of C_μ. In the expressed allele, $V_H/D_H/J_{H4}$ rearrangement was present; however, stop codons existed within the V_H in all potential reading frames. However, the dominant defect was a small insertion/deletion that eliminated the J_H donor splice site. This led to an aberrant RNA splice between the leader region and C_{H1}, creating a shortened μ RNA. A recognition sequence for signal peptidase led to cleavage at the fifth amino acid of C_{H1}. These molecular defects were responsible for the truncated μ chain that lacks a variable region and that fails to assemble a light chain.

The clinical features in γ heavy chain disease vary markedly, ranging from a malignant process with very rapid progression to a course extending over 20 years. The most common presentation is as a lymphoproliferative disorder with hepatosplenomegaly and lymphadenopathy. A partial γ gene deletion has been defined as the molecular abnormality in the two cases examined (Alexander *et al.*, 1982; Guglielmi *et al.*, 1984). In the cDNA prepared from OMM γ3 heavy chain mRNA examined by Alexander and co-workers (1982), the nucleotide sequence was consistent with an extensive internal deletion that encompassed the V region following the first 15 residues as well as the entire C_{H1} domain. The primary synthetic product underwent post-synthetic degradation to yield the circulating heavy chain protein containing an NH_2 terminal deletion with a sequence starting with the hinge. Guglielmi and co-workers (1984) also have examined the molecular defect in a patient with classic γ heavy chain disease in which

an IgG_1 paraprotein lacked the entire variable V_H as well as the first heavy chain constant C_{H1} domain. The paraprotein sequences started in the hinge region at position 211 and proceeded normally through the second and third constant domains. Of note, a short κ mRNA was also present, indicating that the cell possessed two molecular defects. The expressed $C_{\gamma 1}$ gene had undergone a deletional recombination in which material with no obvious switch or variable region information was introduced 5′ to the hinge region, thus eliminating the C_{H1} exon. These data indicate that, in this patient with γ heavy chain disease, a DNA deletion encompassing the $\gamma 1$ C_{H1} exon was present and resulted in an imcomplete γ chain, which begins in the hinge region.

F. T Cell Receptor and Immunoglobulin Gene Defects in Hereditary Primary Immunodeficiency Disorders

The immunodeficiency diseases comprise an array of disorders that are heterogeneous, both in terms of their clinical and immunological manifestations and in terms of their pathogenesis (Bergsma *et al.*, 1975; Cooper and Seligmann, 1977; Horowitz and Hong, 1977; Asherson and Webster, 1980; Rich *et al.*, 1980; Seligmann and Hitzig, 1980; Rosen *et al.*, 1984). Several broad categories of defects have been discerned. First, there are abnormalities involving intrinsic defects in lymphoid cells that are manifest as errors in various steps along the maturation pathways leading from pluripotent hematopoietic stem cells to mature T cells or plasma cells. Second, there are abnormalities in the microenvironments necessary for the generation of differentiation signals essential to the maturation of lymphoid cells; maldevelopment of central lymphoid organs such as the thymus fall into this category. Third, there are disorders of regulatory cells that normally control humoral and cellular immune responses. In this category, one may include disorders of macrophages, as well as abnormalities of helper and suppressor T cells. Finally, there are disorders wherein the production of lymphoid cells and immunoglobulins is normal, but abnormalities of the host environment exist which lead to excessive endogenous catabolism or excessive loss of immune effector elements.

In some cases involving intrinsic defects of lymphoid cells, the underlying deficiency is of critical enzymes, such as those of the purine salvage pathways. Patients with other intrinsic immunological deficiencies are being examined for T cell receptor and immunoglobulin gene defects. For example, patients with the immunodeficiency disease ataxia-telangiectasia manifest breaks and translocations at the sites of the T cell receptor and immunoglobulin heavy chain genes.

Ataxia-telangiectasia is an autosomal recessive, multisystem disorder characterized by cerebellar ataxia, oculocutaneous telangiectasia, recurrent sinopulmonary infections, high incidence of neoplasia, and a variable immunodeficiency state (Peterson *et al.*, 1966; McFarlin *et al.*, 1972; Sedgwick and Boder, 1972; Boder, 1975; Waldmann *et al.*, 1983b). The patients have disorders of antibody responses that are associated with abnormalities of immunoglobulin levels that include an extreme deficiency or absence of serum IgA, IgE deficiency, and reduced serum concentrations of IgG_2 and IgG_4 in most cases. The patients also have various disorders of specific cell-mediated immunity, including an inability to produce antigen-specific cytotoxic lymphocytes to viral pathogens. The patients have an embryonic appearing thymus and persistent production of α-fetoprotein (Waldmann and McIntire, 1972), features suggesting a defect in tissue differentiation. In addition, patients with ataxia-telangiectasia have an increased sensitivity to ionizing irradiation (Gotoff *et al.*, 1967; Cunliffe *et al.*, 1975) and, furthermore, the fibroblasts of patients with ataxia-telangiectasia have a markedly reduced colony-forming ability following X irradiation but are not unduly sensitive to ultraviolet irradiation in contrast to the reciprocal pattern observed in patients with xeroderma pigmentosa (Taylor *et al.*, 1975; Paterson *et al.*, 1976; Kraemer, 1977). There is presumably a defect in DNA post-X-irradiation repair in patients with ataxia-telangiectasia, although convincing evidence for a specific biochemical defect is lacking (Paterson *et al.*, 1976; Taylor *et al.*, 1975; Bridges, 1981). The ataxia-telangiectasia cells examined fail to pause sufficiently after X ray for DNA repair. Rather they launch directly into DNA replication. There is a very high incidence of chromosomal translocations and breaks in T cell lines and leukemias derived from patients with ataxia-telangiectasia that are clustered on chromosomes 14q11, 7p13-15, and 7q32-35 (Cohen *et al.*, 1975; McCaw *et al.*, 1975; Oxford *et al.*, 1975). These are the chromosomal regions that undergo DNA deletion and repair to generate active T cell receptor genes (Murre *et al.*, 1985). Furthermore, site-specific breaks in DNA and the subsequent reconstitution of DNA following gene rearrangement are involved in the generation of active immunoglobulin genes and in immunoglobulin class switch. Patients with ataxia-telangiectasia frequently have breaks and translocations involving chromosome 14q32, the site of the immunoglobulin heavy chain genes. In accord with this observation, the patients frequently have reduced serum concentrations of IgA, IgG_2, IgG_4, and IgE, immunoglobulins that are encoded by genes at the 3′ end of the heavy chain gene cluster (Oxaleus, 1982; Waldmann *et al.*, 1983b). The production of such im-

munoglobulins involves a class switch which again requires DNA deletion and repair. Thus, site-specific DNA breaks involved in the generation of active immunoglobulin and T cell receptor genes may not be normally repaired in patients with ataxia-telangiectasia, an abnormality which may underlie their disorders of specific cellular and humoral immune responses (Waldmann, 1982).

A similar pattern has been observed in patients with another chromosomal instability disorder, the Nijmegen breakage syndrome (Weemales *et al.*, 1981). Siblings with this disorder have been described with microcephaly, mental retardation, stunted growth, café au lait spots, and a broad immunodeficiency involving both T cells and B cells that includes IgA and IgE deficiency. Abnormalities involving chromosome 7 and 14 are found in these patients, with breakpoints clustering at 7p13, 7q35, 14q11, and 14q32, the sites of the T cell receptor and immunoglobulin heavy chain genes.

The humoral immune deficiency diseases may be caused by intrinsic B cell abnormalities or by disorders of regulatory T cells that lead to the absence or marked reduction in the serum concentration of all immunoglobulin classes. Alternatively, the immunodeficiency may involve a single class or subclass of immunoglobulin or a deficiency affecting multiple subclasses but sparing others. In the most common forms of such disorders, congenital hypogammaglobulinemia, common variable hypogammaglobulinemia, selective IgA deficiency, and hyper-IgM associated with absence of other immunoglobulin classes, no immunoglobulin gene defects have been defined to date on examination of the genes encoding the missing subclasses or the regions involved in immunoglobulin class switch. In a number of such cases with familial immunodeficiency, the disordered gene leading to the abnormality has been mapped to a chromosome (e.g., the X chromosome) that does not bear an immunoglobulin gene. In other cases, the abnormal gene has been shown to be unlinked to the immunoglobulin heavy chain gene locus by an approach using an RFLP. Such variation in the location of restriction endonuclease sites in different individuals provides a useful marker to facilitate the mapping of inherited diseases. Two particularly valuable genes with RFLP linked to the immunoglobulin heavy chain genes are the C_μ switch region gene and a gene termed D14S1. This approach has been used to show that certain autosomal recessive forms of familial selective IgA deficiency and of the hyper-IgM syndrome are not linked to the immunoglobulin heavy chain locus.

Stavnezer and Zegers (1986) have demonstrated aberrant κ chain constant region genes in a patient lacking κ light chain-expressing immunoglobulins. Normally, the ratio of κ to λ light chains in serum

immunoglobulins is approximately 2 : 1. In rare cases there have been reports of grossly abnormal κ to λ ratios where this defect must be considered a primary feature of the disease (Bernier *et al.*, 1972; Barandun, 1976; Zegers *et al.*, 1976). λ chain deficiency was described in one patient and κ chain deficiency in three individuals. Clinically, the κ chain-deficient patients included one with recurrent respiratory infections, diarrhea, partial albinism, and a megaloblastic anemia; a second patient with pernicious anemia, achlorhydria, and diarrhea; and a third patient with cystic fibrosis, diabetes, and malabsorption. Stavnezer and Zegers (1986) demonstrated point mutations in both C_κ alleles in one of the patients lacking κ light chains. In one allele there was a point mutation at amino acid position 194 leading to replacement of a Cys by Gly, whereas in the other allele there was a single nucleotide replacement at position 148, resulting in the substitution of Arg for Trp. Both point mutations involve amino acid residues that are required for the formation of the intrachain disulfide bonds of the molecule. The Trp at position 148 which is converted to Arg is a highly invariant residue in all species and appears to shield the intradomain disulfide bond of immunoglobulin domains, thus functioning to stabilize these bonds and, consequently, the folding of the molecule. The other point mutation at amino acid position 194 converted Cys to Gly. This Cys is directly involved in intradomain disulfide bond formation and, thus, this mutation may prevent correct folding of the κ chain. It is not clear whether these two mutations alone result in the complete absence of κ-type immunoglobulin in this patient.

Multiple gene deletions within the human heavy chain cluster have been identified in rare individuals with selective deficiencies of certain immunoglobulin classes. LeFranc and co-workers (1982) reported three healthy Tunisian individuals, two of them brothers, who showed an abnormal immunoglobulin expression with only IgM, IgD, IgG_3, IgE, and IgA_2 present in the serum. Using C_H gene probes, they demonstrated that there had been a large chromosomal deletion that included three γ genes, an α gene, and a pseudo ε gene. This deletion accounted for the simultaneous absence of certain heavy chain subclasses. Similarly, Mingone and co-workers (1984) demonstrated a broad deletion within the heavy chain constant region in two probands of Italian origin. Two different haplotypes were described; the first had deletions of the $C_{\alpha 1}$, $C_{\text{pseudo}\gamma}$, $C_{\gamma 2}$, $C_{\gamma 4}$, and C_ε genes, whereas the second lacked $C_{\text{pseudo}\,\varepsilon}$, $C_{\alpha 1}$, $C_{\text{pseudo}\,\gamma}$, $C_{\gamma 2}$, and $C_{\gamma 4}$ genes. An illegitimate recombination in the germ-line between nonadjacent switch sequences was suggested as a potential explanation for the deletions of the constant region genes observed.

V. Summary

Immunoglobulin and T cell antigen receptor genes in their germline form are organized as discontinuous DNA elements that are joined by recombinations during lymphocyte development. The analysis of immunoglobulin gene structure and arrangement has been of great value in the study of human lymphoid neoplasms. The analysis of rearranged immunoglobulin and T cell receptor genes has been of value in defining the lineage (T or B cell) of neoplasms that were of controversial origin previously, determining the clonality of abnormal lymphocyte proliferations, diagnosing and monitoring the therapy of lymphoid malignancies, determining the state of maturation and the causes for failure of maturation of cells of the B cell series, and providing major insights into the cause of malignant transformation of B and T lymphoid cells. Thus, the application of this molecular genetic approach has great potential for complementing conventional marker analysis, cytogenetics, and histopathology, thus broadening the scientific basis for the classification, diagnosis, and monitoring of the therapy of lymphoid neoplasia.

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ADVANCES IN IMMUNOLOGY, VOL. 40

Human Tumor Antigens

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I. Introduction

Three key developments in biomedical research of the past 25 years led to the current intense interest in human tumor-associated antigens. First was the ever-increasing amount of data indicating that neoplastic transformation is associated with antigenic changes on mammalian cell surfaces. Second was Kohler and Milstein's (1975) method of producing hybridomas that secrete monoclonal antibodies (Mabs). Third was the emergence of modern molecular biology from the combined disciplines of microbiology, genetics, and biochemistry. Precise methods for investigating the structures and functions of nucleic acids and proteins were available by the 1960s but were enormously enhanced by increasingly sophisticated technologies of "gene cloning" during the last decade. Historically, these three advances were preceded by the quest of biomedical scientists to diagnose cancer with simple measurements of "markers" in serum or tissues. This continuous search can be traced from the first tumor marker discovered by Bence-Jones (1847) to the finding by Edelman and Galley (1962) that such Bence–Jones proteins are "markers" of multiple myeloma. The discovery of Abelev *et al.* (1963) of α-fetoprotein, and that of carcinoembryonic antigen by Gold and Freedman (1965), soon followed. A host of other "tumor markers," including numerous enzymes, hormones, serum proteins, and tumor-associated antigens, have been considered potentially suitable for diagnosis at times in the past and continuing into the present. These widespread efforts to develop diagnostic tests for cancer, including the more recent development of radioimmunoscintigraphy to evaluate the type of radionuclide, the mode of conjugation, and the biodistribution of the radiolabeled monoclonal antibody in human tumors have been the subjects of excellent and extensive reviews to which we refer interested readers (Sell and Wahren, 1982; Teh *et al.*, 1985; Sell and Reisfeld, 1985; Foon and Todd, 1986).

This article focuses mainly on those human tumor antigens defined by using murine Mabs as molecular and functional probes. Particular emphasis has been placed on describing those glycoprotein and gan-

glioside tumor antigens that serve as targets for cancer therapy and as biochemically defined cellular markers for ultimately delineating basic mechanisms of tumor invasion and metastasis.

II. Glycoprotein Antigens

Large numbers of glycoproteins have been targeted by murine Mabs as potential human tumor-associated antigens, essentially because of the relative ease by which such Mabs can be produced. However, it is fair to state that the vast majority of these antigens has not been characterized in depth as to their molecular and functional characteristics. On the contrary, the major thrust of these efforts has been to determine the potential of murine Mabs directed to glycoprotein antigens for diagnosis and monitoring of cancer. In most cases, the target antigen specified by the antibodies was thought to be merely incidental and sufficiently characterized by establishing that it was a protein or glycoprotein of a certain molecular weight estimated by SDS–polyacrylamide gel electrophoresis (PAGE) of indirect immunoprecipitates. Concerted efforts were made, however, to develop suitably sensitive assays to detect these antigens in the circulation or in tumor tissues to correlate their incidence with the onset or progression of neoplastic events.

In contrast, the more recent attempts to evaluate the efficacy of Mabs for cancer therapy have involved a real effort, either by coincidence or design, to characterize not only the antibodies but also the molecular and functional properties of their target antigens. Melanoma and colorectal carcinoma-associated antigens have been the most frequent subjects of such research, partly because of the relative ease with which such tumor cells can be established in long-term tissue culture. Therefore, this article will dwell largely on research concerning melanoma and colorectal carcinoma and to a lesser extent with that in neuroblastoma, lung carcinoma, and a variety of other neoplasms.

A. Immunological Characterization

The first report describing murine Mabs directed against human melanoma indicated that these reagents could not only specify different antigenic epitopes but also significantly suppress the growth of human melanoma tumors in athymic mice (Koprowski *et al.*, 1978). Specifically, by fusing BALB/c myeloma cells with lymphocytes from BALB/c mice, primed by intraperitoneal injection of human cultured melanoma cells, hybridomas were produced that secreted antimel-

anoma antibodies that reacted positively in an indirect radioimmunoassay. The existence of different epitopes was apparent when one such antibody reacted with three of six melanoma cells and not with any of the five colorectal carcinoma cell lines or three human fibroblast lines tested. It was possible to use various Mabs to differentiate between melanoma, colorectal carcinoma, and normal human cells, as well as between individual melanomas and colorectal carcinomas. Thus, some antimelanoma antibodies did not cross-react with heterologous tumors whereas others cross-reacted with normal human fibroblasts and with some colorectal carcinoma cells. This study also demonstrated that Mabs could be used to determine the topographical relationship between antigenic determinants by analysis of their competitive or additive binding to a target cell. Antibody combinations delineating different epitopes on melanomas showed additive binding, suggesting sufficient separation of epitopes to prevent steric hindrance.

Dippold *et al.* (1980) selected 18 murine Mabs for their reactivity with cell surface antigens of the immunizing human melanoma cell line SK-MEL-28. Serological assays and absorption analysis defined six distinct antigenic systems. Biochemical analysis indicated that two of the antigens were glycoproteins of M_r 95,000 and 150,000. Two other antigen systems contained heat-labile antigens that could not be defined. The remaining two antigenic systems (R_{24} and O_5) involved ganglioside antigens that are discussed in another part of this article.

A family of antigens of M_r 97,000, designated p97, that is preferentially expressed by human melanoma cells and tissues, has been thoroughly investigated in the laboratories of Karl and Ingegerd Hellstrom and their collaborators. In initial studies, Woodbury *et al.* (1980) described the production and characterization of Mab 4.1 (IgG_1) that bound to a significant extent with approximately 90% of melanomas tested. This same antibody also reacted in binding assays with 55% of a variety of other tumor cells analyzed. However, antibody 4.1 failed to react with several B lymphoblastoid lines or cultivated fibroblasts. The target antigen for this antibody was expressed on both cultured cells and biopsy specimens and proved to be a glycoprotein of M_r 97,000. The investigators emphasized that the widespread occurrence of p97 clearly indicated that it was not melanoma specific. In a follow-up study, immunoperoxidase assays detected no p97 antigen in any of the normal adult tissues examined, whereas five of nine melanoma biopsies showed from low to very high p97 level and three of seven nonmelanoma tumors contained the antigen, although in small amounts. Interestingly enough, p97 was observed in samples of fetal

colon, umbilical cord, and some specimens of fetal lung and benign nevi. This observation led Woodbury *et al.* (1981) to conclude that p97 is a tumor marker of considerable interest. However, they questioned whether the indirect immunoprecipitation method was sufficiently discriminating to detect even small amounts of p97 in normal and neoplastic tissues. In an effort to answer this question, one of their colleagues developed a much improved direct antibody-binding assay (Brown *et al.*, 1981a). Specifically, the ^{125}I-labeled protein A assay for antibody binding to surface antigens of viable cells was used with hybridomas secreting antibodies selected for the IgG_2 isotype to ensure effective binding to protein A. A key feature of the approach by Brown *et al.* (1981a) was to isolate and purify the Mab by affinity chromatography on protein A columns and then to label it with ^{125}I. Results from indirect immunoprecipitation analyses agreed with those obtained by the radiobinding assay and indicated that this immunoprecipitation technique was suitable for rapid and informative screening to identify hybridoma-secreting antibodies reactive with protein antigens of cultured human tumor cells.

Imai *et al.* (1981, 1982) described the immunochemical characterization of four Mabs to human melanoma-associated antigens. These antibodies were shown to be expressed on antigenic structures not associated with β_2-microglobulin or histocompatibility antigens. These findings are in agreement with previous results reported by Carey *et al.* (1976) and McCabe *et al.* (1980) using polyclonal alloantisera and xenoantisera, respectively, to show that serologically detectable human melanoma-associated antigens are not histocompatibility antigens. The antibodies described by Imai *et al.* (1981, 1982) failed to mediate lysis of melanoma cells in the presence of rabbit complement but reacted strongly in rosetting assays with a number of human melanoma cell lines and freshly explanted melanoma cells. In contrast, the antibodies failed to show any significant reactivity with murine B16 melanoma cells or with human B or T lymphoid cells and myeloid cells in long-term culture as well as with fetal, adult, or SV-40-transformed fibroblasts. When testing sera of 87 patients with melanoma at various stages, these investigators were unable to identify any antigens that reacted with one of their Mabs (165.28T).

Morgan *et al.* (1981a) produced and characterized a Mab (9.2.27) to a melanoma-associated antigen that was later identified as a glycoprotein/chondroitin sulfate proteoglycan complex (CSP) (Bumol and Reisfeld, 1982). One of the key features of this work was that the immunogen used to produce this antibody was partially purified from a 4 *M* urea extract derived from M14 melanoma cells by affinity chro-

matography on lentil lectin Sepharose and by removal of fibronectin, a competing immunogen, on gelatin Sepharose. Also important in this approach was a radioimmunometric antibody-binding assay using a chemically defined spent medium of melanoma cells as a solid phase target to screen for suitable antibody-secreting hybridomas. As these investigators noted, their melanoma-associated antigen was on an immunogenic vehicle devoid of competing antigens such as HLA-A,B,C, HLA-DR and fibronectin, and it produced 3.7% of hybrids (from 1590 wells containing hybrids) secreting antibody that bound preferentially to melanoma targets. The antigen defined by antibody 9.2.27 appeared by indirect immunoprecipitation and SDS–PAGE as two components, one of high molecular weight, i.e., >450,000, and the other of 240,000. This molecule was highly sensitive to treatment with trypsin and heat, and although it appeared to be part of the extracellular matrix, it was distinctly different from fibronectin by numerous biochemical criteria (Reisfeld *et al.*, 1982a,b).

It is of interest, as pointed out by Morgan *et al.* (1981a), that their colleagues, Galloway *et al.* (1981), had previously identified an antigen with very similar characteristics by using a polyclonal xenoantiserum. Morgan *et al.* (1981b) subsequently established this antigen as a glycoprotein by intrinsic labeling with several carbohydrate precursors. Later, this same laboratory reported that the antigen recognized by Mab 9.2.27 and considered to have an M_r of 240,000 was associated with a higher molecular weight moiety. Actually they confirmed that a prominent component of M_r 250,000 and a second component of M_r >450,000 were present (Reisfeld *et al.*, 1982a,b, 1984).

Wilson *et al.* (1981) reported on the cellular distribution of melanoma-associated antigens recognized by Mabs (225.28S and 465.12) on cultured human cell lines and skin biopsies by indirect immunofluorescence. The Mab 225.28S was reported to react mainly with a plasma membrane antigen of melanoma cells and nevi consisting of two components of M_r 280,000 and 440,000, whereas antibody 465.12 reacted with tumor tissues of various histological origins and its antigenic target was considered cytoplasmic in nature consisting of four, nondisulfide bridge-linked glycoproteins of M_r 94,000, 75,000, 70,000, and 25,000. In an additional manuscript from this group (Natali *et al.*, 1982), the tissue distribution and molecular profile of the antigen defined by 465.12S were again demonstrated and corroborated.

Several Mabs which identify molecules that appear to be melanoma-associated proteoglycans, at least as judged from their SDS–PAGE profiles, were also reported by Imai *et al.* (1982) and Hellstrom *et al.* (1983). However, none of these investigators clearly determined

that these antigens were proteoglycans but referred to them as "high molecular weight human melanoma associated antigens." When Hellstrom *et al.* (1983) compared SDS–PAGE profiles of immunoprecipitates obtained with their Mab 48.7 to those achieved with Mab 9.2.27, the profiles were identical, i.e., two components of M_r >450,000 and of M_r 250,000, as reported previously by Bumol and Reisfeld (1982). Hellstrom *et al.* (1983) also provided a very thorough binding analysis of Mab 48.7 with a large number and variety of cultured cell lines and of frozen melanoma tissues, showing excellent specificity except for a relatively few metastatic melanoma tissues. Like 9.2.27, their Mab 48.7 also weakly stained some blood vessel cells but no other normal cells or cells from other tumor types.

B. Immunochemical and Molecular Profiles

Mitchell *et al.* (1980, 1981) have delineated the molecular profiles of three distinct human melanoma surface antigens detected by anti-melanoma Mabs. One of these antigens identified by Mab 691-13-17 is the human Ia (HLA-DR) antigen with characteristic α and β subunits. This antibody detects the human Ia antigens on all cell types known to express them, including melanoma, when analyzed in comparison with known anti-HLA-DR antisera. Another antigen was defined by Mab (691-6-37) that reacts with a cell surface, tissue-specific molecule of M_r 80,000 that is not melanoma specific. This antigen was weakly expressed on many different cell lines as demonstrated by surface iodination and intrinsic labeling with glucosamine and fucose. Still, another antigen was specified by Mab 691 I5 Nu-4-B that recognizes a complex of four associated polypeptide chains with M_r of 116,000, 95,000, 29,000, and 26,000, designated α, β, and γ chains, respectively. In their native state, the two smaller chains were covalently linked to a unit of M_r 116,000 by disulfide bonds. The polypeptide chain of M_r 95,000 was apparently attached by noncovalent interactions and cleavage of the two major polypeptides by 2-nitro-5-thiocyanobenzoic acid and subsequent peptide mapping revealed distinct patterns of cleavage, suggesting that these two proteins have different primary amino acid structures.

In a structural characterization of the melanoma-associated antigen p97, Brown *et al.* (1981b) were able to demonstrate by sequential immunoprecipitation and SDS–PAGE that four different Mabs recognized the same p97 molecule. Mabs 4.1 and 8.2 reacted with the same antigen epitope p97a, whereas Mabs 118.1 and 8.2 defined epitopes p97b and p97c, respectively. Moreover, these authors found that six additional Mabs previously reported to be specific for a melanoma cell

surface protein of M_r 95,000 (gp95) (Dippold *et al.*, 1980) also bound to p97 indicating that p97 and gp95 were identical. Brown *et al.* (1981b) point to the utility of immunoprecipitation and SDS–PAGE as alternative screening methods for hybridomas secreting antibodies directed to different epitopes of the same antigen molecule. Since only relatively few distinct proteins, ranging usually from only 5 to 10, were recognized in any one fusion, it seemed advantageous to vary the immunizing melanoma line. It was somewhat surprising that such a relatively high frequency of hybridomas secrete antibodies to p97. However, p97 may be particularly immunogenic or simply expressed in larger amounts than other antigens on the cell lines used for immunization (K-2 and SK-MEL 28). Quantitation showed that SK-MEL 28 melanoma cells express at least 400,000 molecules of p97 per cell based on antibody binding data, i.e., roughly equal to the number of HLA-A,B,C determinants identified by binding of anti-HLA Mab W6/32 to these same cells.

Regarding the expression of epitopes p97a, p97b, and p97c on different cell types, Brown *et al.* (1981b) showed that a melanoma, a lung carcinoma, as well as a B and T lymphoid cell line all bound each antibody defining these epitopes but in vastly differing amounts, i.e., the melanoma line bound 300,000 to 380,000 molecules of each antibody per cell; the lung carcinoma line bound 4000 to 6200 molecules per cell; whereas the B and T cell lines bound only 250 to 1400 molecules/cell.

The initial chemical characterization of p97 indicated that it contained sialylated carbohydrate chains and consisted of a single polypeptide, most likely with some intrachain disulfide bridges. Partial digestion of detergent-solubilized p97 with either papain or trypsin produced seemingly identical fragments of M_r 40,000 that were glycosylated and contained the p97a,b,c epitopes (Brown *et al.*, 1981b).

Brown *et al.* (1982) also reported that their human melanoma-associated antigen p97 is functionally related to transferrin and that the two proteins evolved from a common ancestor. They based their conclusion on the amino-terminal amino acid sequence homology between these 2 molecules, i.e., 7 of 12 residues determined were identical. This includes two of the least common amino acids, i.e., tryptophan and cystine. They supported their conclusions by finding that an antiserum to denatured p97 cross-reacted with denatured transferrin and lactotransferrin and also by data indicating that p97 bound iron.

However, p97 is not the transferrin receptor although it shares a similar molecular weight. In fact, Brown *et al.* (1982) showed conclusively that these two molecules are not identical. In fact, upon SDS–

PAGE under nonreducing conditions, transferrin receptor formed a dimer of M_r 200,000, whereas p97 migrated slightly faster than the reduced protein. In addition, the tissue distribution of the two molecules differed considerably and Mab OKT9, which is specific for transferrin receptor, did not immunoprecipitate p97 from radioiodinated melanoma cells. The immunological difference of these two molecules was further demonstrated by sequential immunoprecipitation experiments. Finally, p97 also failed to bind to a transferrin–Sepharose column. Although p97 and transferrin receptor are structurally distinct, Brown *et al.* (1982) proposed that they may share some biological functions, e.g., mediation of cellular uptake of transferrin-bound iron. This hypothesis lacks experimental proof. Brown *et al.* (1982) also admit that in view of the trace amounts of p97 in normal adult tissue, it appears more likely that this function is carried out by transferrin receptor rather than by the p97 molecule. It seems clear that the distribution of p97, which is more or less restricted to melanoma cells, nevi, and fetal intestine, argues for a more specialized functional role of this molecule, which may be restricted to these tissues. This contention remains to be substantiated.

In an extensive pioneering effort, Rose *et al.* (1986) have purified and cloned the mRNA of the p97 antigen and determined its complete nucleotide sequence. From this sequence, the amino acid sequence of the 738-residue p97 precursor was deduced. Concerning its overall structure and possible functional domains, after removal of a 19-residue signal peptide, the mature p97 molecule contains 2 extracellular domains of 342 and 352 residues. A C-terminal 25-residue stretch, composed mainly of uncharged, hydrophobic amino acids, is thought to be a membrane anchor for the p97 antigen. There is considerable folding in this molecule as well as a good deal of sequence conservation as each of the extracellular domains contains 14 cysteine residues that form 7 intradomain disulfide bridges. Interestingly enough, the p97 molecule is not heavily glycosylated since extracellular domain contains only one or two potential N-glycosylation sites. The three major antigenic determinants appear to be protein, and all are present on the N-terminal domain of the p97 molecule. Not too surprisingly, when viewed on the basis of previous reports published by Brown and colleagues (1982, 1985), the two domains show 39% homology to the corresponding domains of human serum transferrin and also have a 46% amino acid homology to each other. Rose *et al.* (1986) suggest that conservation of disulfide bridges and of amino acids, thought to compose the iron-binding pockets, constitutes further proof that p97 is also related to transferrin in tertiary structure and function. In this

regard, Brown *et al.* (1982) previously showed that p97 binds iron, but not nearly to the same extent as transferrin. It is certainly interesting to contemplate, as done by Rose *et al.* (1986), that based on the structural homologies between p97 and the three members of the transferrin super family, p97 may have diverged from serum transferrin more than 300 million years ago when mammalian and avian lineages diverged. One must wonder about the functional role of this membrane-bound, transferrin-like molecule in neoplastic cells where it may well differ considerably from the role played in cellular iron metabolism by circulating serum transferrin and transferrin's cellular receptor. In a recent study, Rose *et al.* (1986) propose the name melanotransferrin for the p97 molecule to denote its original identification. Even though this molecule is represented on tumor cells other than melanoma, this name is certainly appropriate. Moreover, these investigators deserve more recognition than received thus far, since this is one of the first detailed analyses of the primary structure of a complex, human, membrane-bound tumor-associated antigen.

Imai *et al.* (1982) described an antigen expressed on both melanoma and carcinoma cells detected by another Mab, 376.96S. In contrast to the antigen they described previously (Wilson *et al.*, 1981), which was recognized on tumor cells of varied histological origins by Mab 465.12S and consisted of four glycopeptide chains, the new-found antigen had only a single glycoprotein chain of M_r 94,000, although it is also expressed on a variety of melanoma and carcinoma cells. Nevertheless, 376.96S differed from other antibodies recognizing the antigen p95 described by Dippold *et al.* (1980) and is identical to the p97 antigen of Brown *et al.* (1981b). Additionally, Mab 376.96S distinguishes another melanoma-associated antigen of similar molecular weight that was originally reported by Mitchell *et al.* (1980). Like Mab 465.12S, 376.96S also mediates both complement-dependent and cell-dependent lysis of melanoma and carcinoma cells.

A unique glycoprotein–proteoglycan complex associated with human melanoma cells was defined with Mab 9.2.27 by Bumol and Reisfeld (1982). Biosynthetic studies demonstrated that this complex consisted of an N-linked glycoprotein of M_r 250,000 and a high-molecular-weight proteoglycan component in excess of M_r 450,000. By pulse–chase analysis, the M_r 250,000 glycoprotein was shown to have endo-β-*N*-acetylglucosaminidase H (Endo H)-sensitive precursor, i.e., *N*-asparagine-linked, unprocessed, high mannose oligosaccharides. Additional experiments indicated that the molecule of M_r 250,000 was actually the core glycoprotein of the proteoglycan and its selective digestion with chondroitin lyases indicated that it was a

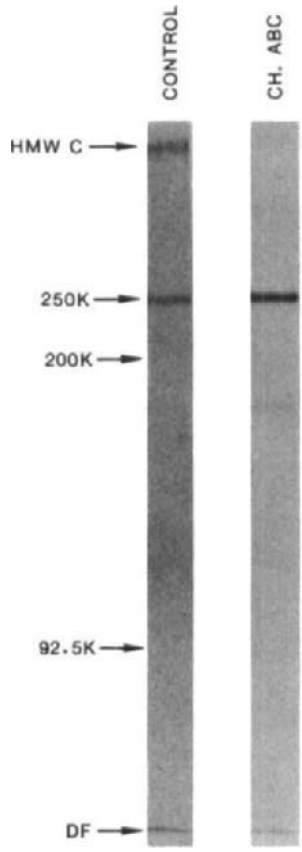

FIG. 1. SDS-PAGE profile of a detergent extract from M21 melanoma cells, intrinsically labeled with [^{3}H]valine, following indirect immunoprecipitation with Mab 9.2.27. The profile on the right indicates the effect of treating the cell extract with chondroitinase lyase ABC that cleaves chondroitin sulfate chains from the intact proteoglycan of M_r > 450,000 (HMWC) leaving only the core glycoprotein of M_r 250,000. The profile on the left (control) is that of the same detergent extract not digested with chondroitinase lyase ABC. DF signifies dye front.

CSP (Fig. 1). This was later verified by glycosaminoglycan analyses in the same laboratory (Harper *et al.*, 1984; Bumol *et al.*, 1984). Bumol and Reisfeld (1982) also demonstrated that the cationic ionophore monensin drastically affected, i.e., blocked biosynthesis of the proteoglycan and that the appearance of the proteoglycan was linked kinetically to biosynthetic functions of the Golgi apparatus, the proposed site for the glycosyltransferases involved in proteoglycan biosynthesis. These data taken together suggest that the chondroitin sul-

fate chains are added onto the M_r 250,000 core glycoprotein to form a M_r > 450,000 proteoglycan.

Subsequently, Bumol *et al.* (1984) demonstrated in pulse–chase experiments that Mab 9.2.27 recognized several N-linked glycosylated precursor components of M_r 210,000 and 220,000, as judged by their sensitivity to Endo-H, in addition to another precursor of M_r 240,000 and the 250,000 core glycoprotein. The pulse–chase data indicate that the addition of N-linked oligosaccharides is an early biosynthetic event in CSP core protein biosynthesis and that the early antigens of M_r 210,000, 220,000, and 240,000 recognized by Mab 9.2.27 chase into an M_r 250,000, Endo H-resistant glycoprotein and a high-molecular-weight (>450,000) CSP whose only appearance in immunoprecipitates coincides with the acquisition of Endo H-resistance by the M_r 250,000 component. Data from this pulse–chase/indirect immunoprecipitation analysis are shown in Fig. 2.

Tryptic peptide maps of the [^{3}H]arginine-labeled core glycoprotein of M_r 250,000 and CSP recognized by Mab 9.2.27 revealed close structural homology, indicating that the protein portion of the CSP is represented by the M_r 250,000 core glycoprotein. Further studies indicated that Mab 9.2.27 recognizes intact extracellular CSP and that the CSP contains both ΔDi-4S and ΔDi-6S chondroitin sulfate disaccharides. Bumol *et al.* (1984) also demonstrated in long-term pulse–chase experiments that cell-associated, core glycoprotein of M_r 250,000 was detectable 48 hours after a 10-minute biosynthetic pulse, indicating the availability of a long-lived intracellular pool of this glycoprotein for CSP biosynthesis. Cell-associated CSP observed even 72 hours after a 10-minute biosynthetic pulse were thought to be the result of *de novo* synthesis from core glycoprotein pools. It was also considered possible that certain cell surface CSPs are long lived at the melanoma cell surface. As far as the possible functional roles of CSPs in human melanoma are concerned, Bumol *et al.* (1984) utilized Mab 9.2.27 as a probe to investigate the effect of masking these determinants when melanoma cells are interacting with basement membranes. Results from such studies indicated that Mab 9.2.27 can block the early events of melanoma cell spreading on endothelial basement membranes while only slightly inhibiting cell adhesion, suggesting that CSPs may play a role in stabilizing cell substratum interactions in this *in vitro* model for metastatic invasion. Harper and Reisfeld (1983) demonstrated another potential functional role of CSP when they showed that Mab 9.2.27 specifically inhibited anchorage-independent growth of human melanoma cells in a soft agar clonogenic assay *in vitro*. The data of Bumol and Reisfeld (1982), Bumol *et al.* (1984), and Harper

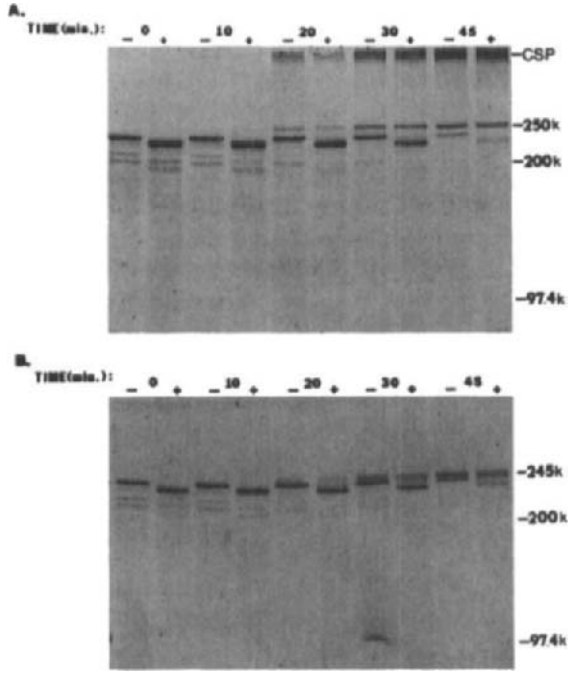

FIG. 2. Pulse–chase/indirect immunoprecipitation analysis of antigens recognized by Mab 9.2.27. Detergent extracts of [^{35}S]-methionine pulse-labeled M21 melanoma cells were immunoprecipitated with Mab 9.2.27 at the time points indicated in the chase and were then either analyzed as control profile (− lanes) or digested with endo-β-*N*-acetylglucosaminidase (+ lanes) before analysis on SDS–PAGE (5% polyacrylamide) (A). A parallel and identical pulse–chase experiment with M21 cells preexposed to 10^{-7} *M* monensin is shown in (B). CSP, Chondroitin sulfate proteoglycan; migration of molecular weight standards (200K, 97.9K) is indicated in this 6-day fluorograph exposure.

and Reisfeld (1983) clearly show that Mab 9.2.27 is an effective probe for biosynthetic, topographical, and functional studies of CSP and its core glycoprotein. This reagent should improve our basic understanding of noncartilage proteoglycans and their potential roles in the biology of metastatic human melanoma.

Albino *et al.* (1983) performed a biochemical analysis of a glycoprotein antigen of M_r 130,000 (gp130) expressed on human melanoma cells. This antigen was first described by their co-workers (Dippold *et al.*, 1980) after analyzing mouse Mabs prepared against the human melanoma cell line SK-MEL-28. Serological analyses by direct immunoprecipitation analysis and blocking tests with $(Fab')_2$ fragments done by Albino *et al.* (1983) indicated that eight Mabs to gp130 distin-

guished at least four spatially distinct epitopes on the exposed surface of this molecule. Pulse–chase biosynthetic studies revealed that gp130 was synthesized via a glycosylated precursor formed from non-glycosylated protein of M_r 80,000. Based on these data, gp130 was thought to contain as much as 40% carbohydrate. Since the M_r 100,000 precursor incorporated glucosamine and was susceptible to digestion with Endo H, presumably the oligosaccharide chains are of the high mannose type. Based on pulse–chase data, it is proposed that conversion to the complex-type oligosaccharides chains of the gp130 molecule may occur quickly once the precursor of M_r 100,000 has reached the Golgi (Albino *et al.*, 1983).

Ross *et al.* (1983) isolated a melanoma-associated proteoglycan (MPG) and characterized it by chemical means. This antigen, termed O-95-45 since it was specifically recognized by a Mab of this designation, was purified by immunoaffinity chromatography from detergent extracts of human melanoma cells (WM 164) after the extracts underwent several gel filtration procedures. The antigen was concentrated and separated from interfering low-molecular-weight compounds by repeated precipitation with acetone at 0°C. The antigen's two components were a CSP of $M_r > 500{,}000$ and a core glycoprotein of 260,000, p*I*6.9. Although the CSP contained both N- and O-linked carbohydrates, the core glycoprotein had only N-linked carbohydrate. The antigenic determinant recognized by Mab 0-95-45 was represented on both components and was heat sensitive, suggesting a protein nature. Since chondroitinase converted the CSP to a molecule of M_r 260,000, the protein cores of the two components appeared to be the same or similar. Sequence analysis could not be done since the N terminus was blocked, a fact which cannot be attributed to the solvent treatment during isolation since myoglobulin subjected to the identical procedure maintained its free N terminus. The amino acid composition of the antigen was relatively polar with an unusually high leucine and low lysine content and, curiously enough, when compared to published amino acid compositions of cartilage type proteoglycans, it most closely resembled that of a heparan sulfate proteoglycan (Ross *et al.*, 1983).

Mab 155.8 produced against purified membranes of human melanoma cells was reported to react with a CSP preferentially expressed on such cells. Binding inhibition studies indicated that Mab 155.8 reacted with an epitope different from that recognized by Mab 9.2.27 on the same proteoglycan molecule (Harper *et al.*, 1984). Mab 155.8 bound melanoma cells to a lesser extent than Mab 9.2.27, suggesting either that the former antibody recognized determinant(s) found in

smaller numbers on the cell surface or that there was a difference in the affinity constants of the two antibodies. The difference in reactivities of these two Mabs with CSP is underlined by results from immunodepletion analysis, which showed that 155.8 determinants were present on only a subgroup of those molecules bearing the 9.2.27 epitope.

The proteoglycan nature of the molecules defined by Mab 155.8 was clearly evident from data of [^{3}H]leucine incorporation into the high-molecular-weight CSP of >450,000 and analysis of $^{35}SO_4^{2-}$-labeled material by cellulose acetate electrophoresis following elimination of O-linked glycosaminoglycans by alkaline borohydride treatment. Data from this analysis along with chondroitinase ABC sensitivity confirmed that the sulfated glycosaminoglycans associated with antigen(s) identified by Mab 155.8 indeed contained chondroitin sulfate type A and/or C. Similar to 9.2.27, Mab 155.8 also recognized determinants on the M_r 250,000 core glycoprotein and the intact CSP. The disappearance of CSP and a concomitant increase in concentration of the component of M_r 250,000 following chondroitinase digestion of immunoprecipitated proteoglycans proved that this glycoprotein was included in the CSP and was, in fact, its core protein. Harper *et al.* (1984) provided direct evidence that Mabs 9.2.27 and 155.8 recognize the proteoglycan in the absence of its core glycoprotein, thus ruling out the possibility that the proteoglycan contained no antigenic determinants but was complexed only with the core glycoprotein and merely a "passenger" in immunoprecipitates. Specifically, after resolving antigens extracted from human melanoma cells by $CsCl_2$ density centrifugation in the presence of detergent and high salt content, it became possible to immunoprecipitate high-density proteoglycans alone as well as the free M_r 250,000 core glycoprotein from lower density fractions of the gradient.

The topographical distribution of Mab 155.8-defined proteoglycans on the surfaces of paraformaldehyde-fixed human melanoma cells was examined by indirect immunofluorescence, revealing filamentous structures that sometimes connected cells with the underlying substratum. These molecules were not found as substrate-attached material left behind when cells were removed. This raises interesting questions as to the involvement of CSP in melanoma cell adhesion and spreading on various substrata. In this regard, there is some evidence indicating that Mabs 9.2.27 and 155.8 only minimally affect adhesion, but inhibit to a greater extent cytoplasmic spreading of human melanoma cells on plastic and collagen–fibronectin complex substrata (Harper *et al.*, 1984).

The finding of a Mab 155.8-defined CSP on freshly explanted melanoma tissues by indirect immunoperoxidase techniques underlines its functional relevance. In fact, the absence of the proteoglycans defined by Mabs 9.2.27 and 155.8 in normal fetal and adult tissues suggests that determinants recognized by these antibodies are not found in normal tissues known to be rich in CSP, i.e., cartilage (Harper *et al.*, 1984). Judging from the data of Ross *et al.* (1983), Bumol and Reisfeld (1982), Bumol *et al.* (1984), and Harper *et al.* (1984, 1985, 1986), all their respective MPGs appear to be similar if not identical. In this regard, Ross *et al.* (1983) demonstrated that Mab 9.2.27 developed by Morgan *et al.* (1981a) effectively inhibited the binding of Mab 0-95-45 and reacted with very similar heat and trypsin-sensitive antigenic determinants, immunoprecipitating essentially the same molecular species of proteoglycans. Ross *et al.* (1983) advanced some interesting hypotheses as to cell surface expression and molecular interactions of the component molecules of the proteoglycan detected by Mab 0-95-45 and as to its possible heparan sulfate proteoglycan nature. However, it is now abundantly clear from a wealth of experimental data that at least Mab 9.2.27 detects a melanoma-associated CSP (Bumol and Reisfeld, 1982; Harper *et al.*, 1984; Bumol *et al.*, 1984). This Mab is also an excellent probe to delineate the biosynthesis, intracellular transport, and pathways to cell surface expression of this proteoglycan molecule, as recently confirmed by Spiro *et al.* (1986).

C. Biosynthesis and Intracellular Transport

In a follow-up study designed to define chemically and immunochemically the rather unique antigen on human melanoma cells recognized by Mab 9.2.27, Bumol and Reisfeld (1982) used a combination of biosynthetic and enzymatic methods. They propose that Mab 9.2.27 recognizes both the free pool of core protein and the CSP monomer. Bumol and Reisfeld (1982) confirmed by tryptic peptide map analysis and chondroitinase lyase AC and ABC digestion that the molecule of M_r 250,000 is indeed the core protein of the CSP and simply has glycosaminoglycans added as side chains. As stated earlier, the cationic ionophore monensin effectively blocks the appearance of the $M_r > 450{,}000$ proteoglycan in immunoprecipitates obtained with Mab 9.2.27 from detergent extracts of intrinsically radiolabeled melanoma cells that were previously exposed for 18 hours to 10^{-7} *M* monensin. This finding correlates with that reported by Tajri *et al.* (1980) indicating that monensin can effect the biosynthesis of proteoglycans in chondrocytes. Pulse–chase analyses of the Endo H-treated antigen complex also suggested that the appearance of the proteoglycan is

kinetically linked to biosynthetic functions of the Golgi apparatus, the site proposed for biosynthesis of proteoglycans involving glycosyltransferases (Roden, 1980). Bumol and Reisfeld (1982) interpret the unique specificity of Mab 9.2.27 for an M_r 250,000 N-linked, sialylated core glycoprotein associated with a common CSP as suggesting that the human melanoma cell may actually express modified or even unique gene products capable of serving as acceptors or core glycoproteins for common proteoglycan oligosaccharide side chains. If so, this type of alteration may account for the previously reported changes in proteoglycans of the membranes and extracellular matrix of some tumor cells (Heaney-Kleras and Kleras, 1980; Glimelius *et al.*, 1978).

Bumol *et al.* (1983) also investigated antigenic molecules recognized by Mab F11. By indirect immunoprecipitation analysis, this antibody recognizes a glycoprotein of M_r 100,000 in the spent medium of M14 melanoma cells. A synthetic, serum-free medium was used for these cultures. However, a parallel analysis of detergent extracts of these M14 cells reveals a more complex profile—three glycoprotein molecules of M_r 75,000, 77,000, and 100,000. Pulse–chase studies indicate that Mab F11 initially recognizes the molecules of M_r 75,000/77,000 early in the chase in the absence of detectable antigen of M_r 100,000. Since the M_r 75,000/77,000 components increase in intensity until the first appearance of the M_r 100,000 component, which occurs 180 to 240 minutes into the chase, it is likely that they are biosynthetic precursors of the larger molecule. Therefore, it is unlikely that Mab F11 simply recognizes a common antigenic site on three distinct glycoproteins. Bumol *et al.* (1982) propose that common antigenic sites can exist on different molecules within the same cell and that they may differ structurally as to their biosynthetic intermediates. Their contention is based on data showing sensitivity to degradation of the M_r 100,000 component by Endo H, thus demonstrating that the *N*-asparagine-linked oligosaccharides of this glycoprotein antigen are not processed by the Golgi apparatus. Since this organelle is considered to be the site of terminal glycosylation of *N*-asparagine-linked oligosaccharides, as well as that of enzymes involved in O-linked oligosaccharide biosynthesis, considerable glycosylation may still occur in the Golgi apparatus to form the M_r 100,000 molecules starting from a lower molecular weight 75,000/77,000 core antigen, with the antigen of M_r 100,000 destined for secretion into the extracellular milieu of melanoma cells. The sole addition of sialic acid residues cannot account for all the biosynthetic modifications observed, because treatment of the M_r 100,000 component with neuraminidase reveals a desialylated molecule of M_r 89,000. Studies with 72-hour

fresh surgical melanoma explant cultures indicated that the components of M_r 77,000 and 100,000 recognized by F11 are also synthesized by these primary tumor cultures, suggesting a strong *in vitro/in vivo* correlation with antigens recognized by Mab F11.

Harper *et al.* (1985, 1986) used Mab 9.2.27 as a molecular and functional probe to demonstrate that the core protein of the MPG recognized by this antibody is present in two forms on the cell surface, either free or modified by the addition of chondroitin sulfate chains. These findings suggest for the first time that the addition of glycosaminoglycan chains may not be a prerequisite for cell surface expression of the proteoglycan core protein. Additionally, these authors found that free core protein expressed on the melanoma cell surface apparently is not a consequence of an overflow of the proteoglycan synthetic pathway, since experiments using a β-D-xyloside acceptor of chondroitin sulfate chains indicated the core protein to be a limiting factor in proteoglycan synthesis. Treatment of human melanoma cells with dilute NH_4Cl inhibits the synthesis of melanoma-type proteoglycans and produces a shift in the balance of surface core protein toward the free form. The authors postulate that vesicular acidification may be required to transport core protein molecules through intra-Golgi sites for glycosaminoglycan addition prior to their cell surface expression. Harper *et al.* (1985, 1986) point out that treatment of melanoma cells with dilute NH_4Cl, which is known to disrupt intracellular pH gradients, as well as mechanisms requiring vesicular acidification, may actually limit the synthesis of MPG without much affecting the cell surface expression of its core protein. This contention is supported by the authors' data, indicating that NH_4Cl treatment results in an accumulation of free core protein on the cell surface without a concomitant decrease in the amount of surface MPG. Moreover, the inhibition of proteoglycan synthesis after NH_4Cl treatment cannot be attributed to disruption caused by enzymes and precursors involved in glycosaminoglycan synthesis, since cells treated in this manner retain their ability to initiate and elongate chondroitin sulfate chains on a β-D-xyloside acceptor. In contrast to the more selective action of NH_4Cl, the cationic ionophore monensin inhibits both core protein maturation and synthesis of glycosaminoglycan chains.

Harper *et al.* (1985, 1986) raise some interesting questions with regard to the function of glycosaminoglycan chains in view of the expression of the MPG core protein on the cell surface as a free glycoprotein as well as in the form of a CSP. They point out that, since the addition of these chains is not a prerequisite for cell surface expression of the core protein, it is unlikely that such a modification serves as

a marker to segregate molecules on the cell surface. Signals involved in targeting MPG or its core protein to the cell surface may more likely reside in the N- and/or O-linked oligosaccharides that are added to the core protein prior to addition of glycosaminoglycan chains. However, the authors believe that the addition of chondroitin sulfate chains to the core protein of MPG may well relate to the function of this molecule in the biology of melanoma cells. Previous studies by Harper and Reisfeld (1983) implicated the MPG in processes important for the metastasis of melanoma tumor cells *in vivo*. The findings by Harper *et al.* (1985, 1986) indicating that both the M_r 250,000 core protein and MPG are expressed on the surfaces of melanoma cells leave one with the question as to which is the biologically functional form. As pointed out by these investigators, glycosaminoglycans on the core protein could either render this molecule inactive or, alternatively, constitute the functional determinant necessary for biological activity. Although additional studies are required to answer this question, a well-defined reagent such as Mab 9.2.27 used in conjunction with modern biotechnological methods at least serves to raise such key issues and may, indeed, eventually resolve some of them.

Spiro *et al.* (1986) have used Mab 9.2.27 as a molecular probe to examine in some depth the assembly, transport, and cell surface expression of human melanoma-associated CSP. This was accomplished by pulse–chase experiments and pretreatment of human melanoma cells with diethylcarbamazine (DEC), an inhibitor of proteoglycan biosynthesis in rat chondrosarcoma cells. In comparing their results with those obtained earlier by Harper *et al.* (1985, 1986) after using NH_4Cl to inhibit the synthesis of melanoma-type proteoglycan, Spiro *et al.* (1986) demonstrate that DEC inhibits synthesis and cell surface expression of this proteoglycan at a site distinct from that affected by NH_4Cl, although both agents perform this task in a dose-dependent manner. The usefulness of Mab 9.2.27 as a molecular and functional probe was shown when this antibody clearly distinguished different molecular forms of the proteoglycan core protein as seen in SDS–PAGE of melanoma cell detergent lysates. Specifically, this core protein was recognized as a molecule of M_r 250,000 in the melanoma-associated CSP; however, only a Endo-H-sensitive intermediate of M_r 240,000 was detected by Mab 9.2.27 inside melanoma cells treated for 60–120 minutes with 10–15 m*M* DEC. When human melanoma cells were incubated for 10 minutes with 15 m*M* DEC and [^{35}S]methionine (100 μCi/ml), washed and chased for 4 hours in radioactive-free medium, the intermediate of M_r 240,000 slowly converted to an Endo-H-resistant intermediate of M_r 250,000 but not to a mature, glycos-

aminoglycan-substituted proteoglycan. Only a small amount of this intermediate was transported to the plasma membrane within 5 hours in the presence of DEC. In contrast, most of the peptide core synthesized by melanoma cells in the presence of NH_4Cl was converted to a resistant intermediate of M_r 245,000 detectable on the cell surface. Changes at the ultrastructural level that occurred when melanoma cells were treated with DEC included the appearance of large vacuoles and distension of the Golgi and endoplasmic reticulum. NH_4Cl-treated melanoma cells exhibited fewer vacuoles than DEC-treated cells, although more than normal cells. Taken together, the findings by Spiro *et al.* (1986) indicate that DEC inhibits vesicular transport of translated peptide core from endoplasmic reticulum to Golgi and from there to the plasma membrane of human melanoma cells.

D. Preclinical Models for Immunotherapy

1. In Vitro Studies

Steplewski *et al.* (1979) reported that Mabs against human melanoma-associated antigens mediated antibody-dependent cell-mediated cytotoxicity (ADCC) against melanoma cells *in vitro*. The oftenmentioned "antigenic drift" on tumor cells apparently did not influence these reactions, since they occurred equally well with 2 freshly established melanoma lines and with tumor cell lines maintained in culture for more than 100 passages. However, the antigens evident in ADCC were not found on skin fibroblasts of the patients from whom the melanomas were obtained. In general, the data showed that antibody cytotoxicity mediated by mouse effector cells was considerably less than that by human effector cells. The authors believe that the reactivity observed in ADCC may be directed in part against HLA-DR alloantigens expressed on melanoma target cells. Contrary to this argument is the fact that one Mab produced by immunizing mice with a somatic cell hybrid formed between melanoma and mouse fibroblasts did not react with HLA-DR antigens and thus might have specifically recognized melanoma tumor-associated antigens in both ADCC and a radioimmunoassay. Although the authors stated that the number of samples in their study was too small for them to make definitive conclusions, their results strongly suggested that suppression of tumor growth in nude mice resulted from interactions between "killer cells" and Mabs, especially since the latter were cytolytic in the presence of complement (Steplewski *et al.*, 1979).

Hellström *et al.* (1981a) found 2 murine IgG_{2a} Mabs, 96.5 and 118.1, directed to different epitopes of p97, that acted synergistically in com-

plement-dependent cytotoxicity with respect to 10 of 12 melanoma cell lines tested. In other words, this cytotoxicity was much greater in the presence of both antibodies than with either antibody alone. This effect was specific, since it was not detected when these two antibodies were tested in combination with an IgG_{2a} antibody directed to a different cell surface glycoprotein or against four nonmelanoma cell lines. Whether the synergistic tytotoxic effects of the two antibodies can be used therapeutically is unknown, especially since p97 is expressed on a variety of normal human cells. Additionally, of course, these mouse IgG_{2a} Mabs would have to fix human complement to a significant extent.

Yeh *et al.* (1981) reported the existence of clonal variation in the expression of a Mab-defined human melanoma antigen. This determinant, designated 3.1, was expressed on tumors consisting of mixed populations of antigen-positive and antigen-negative cells. Testing with a variety of serological techniques and cloning short-term human melanoma explants M1801 and M1804 disclosed that the 3.1 antigen disappeared from some of the cells that originally expressed it. These investigators suggest that genetic mechanisms such as chromosomal deletions may be responsible for this phenomenon. From a practical point of view, loss of a determinant such as 3.1 may illustrate a possible complication resulting from the use of just one Mab for immunotherapy. This problem can be overcome by combining multiple Mabs directed to different antigen epitopes of the same molecule, as discussed in some detail by Lane and Koprowski (1982).

Hellstrom *et al.* (1981b), in examining functional activity of a Mab-defined melanoma antigen, found that Mab 3.2 of the IgG_{2a} isotype that defines the 3.1 antigen mediated ADCC of human melanoma cells expressing this antigen and that lymphocytes from human peripheral blood acted as effector (killer) cells. In contrast, Mab 3.1, which is of IgG_1 isotype and directed to the same 3.1 antigen as the IgG_{2a} antibody 3.2, evoked only weak and inconsistent ADCC. These observations certainly confirm the findings of Herlyn *et al.* (1979) and extend them in one important aspect: human peripheral lymphocytes can function equally well or better than mouse splenocytes as a source of effector (killer) cells in the presence of mouse Mabs. These findings also indicate that different classes of immunoglobulins vary in their ability to evoke ADCC.

Proteoglycans have been implicated in growth control, cell–substratum interaction, and other functional properties of potential relevance to tumor metastasis (Culp *et al.*, 1979). In an attempt to gather additional evidence in this area, Mab 9.2.27 was used as a specific

probe to delineate the possible role of proteoglycans in cell–cell interactions and growth control of human melanoma cells (Harper and Reisfeld, 1983). Mab 9.2.27 inhibited anchorage-independent growth of human melanoma cells in soft agar, an event that had been shown to correlate with tumorigenicity *in vivo* (Shin *et al.*, 1975; Harper and Reisfeld, 1983). Data obtained from the double agar clonogenic assay indicated that growth inhibition by 9.2.27 in this *in vitro* system was indeed specific (Harper and Reisfeld, 1983). Thus, monoclonal anti-HLA-A,B,C Mabs that bound to human melanoma cells had no significant effect on their plating efficiency and Mab 9.2.27 also did not affect the plating efficiencies of two 9.2.27 antigen-negative cell lines.

The precise mechanism underlying this phenomenon is not entirely clear. Harper and Reisfeld (1983) postulate that it relates to the physicochemical nature and location of the proteoglycan antigen recognized by Mab 9.2.27 on the surfaces of human melanoma cells. In this regard, it was shown that human tumor cells maintained on an extracellular matrix exhibited higher growth rates and had lower serum requirements than identical cells grown on plastic (Gospodarowicz *et al.*, 1978; Vladavsky *et al.*, 1980). It is believed that the major components of the extracellular matrix, i.e., collagens, laminin, fibronectin, and proteoglycans, work in concert to produce this effect (Gospodarowicz *et al.*, 1978). Although human melanoma cells lack an organized extracellular matrix (Bumol *et al.*, 1984), immunoperoxidase analysis of freshly explanted melanoma tumor tissue showed that the cells synthesized and deposited CSP pericellularly among other matrix components *in vivo* (Harper *et al.*, 1982). Thus, when melanoma cells are grown in soft agar containing Mab 9.2.27, cell–cell interactions may actually be disrupted by this antibody although the pericellular disposition and organization of the proteoglycans remain; consequently, Mab 9.2.27 may be involved in interactions important for the growth of melanoma cells in an anchorage-independent fashion. There are apparently no metabolic constraints on melanoma cells that are attached and spread on a solid substratum, since the binding of Mab 9.2.27 to melanoma cells grown in liquid culture affected neither DNA nor protein synthesis even after 3 days. These data of Harper and Reisfeld (1983) strongly suggest that CSP may be among those molecules on the surfaces of human melanoma cells that are involved in cell–cell interactions important to anchorage-independent growth regulation. Studies of human tumor metastasis, such as these with specific Mab probes, should provide a better understanding of the molecules involved in tumor growth and may eventually lead to more effective treatment and ultimate prevention of metastasis.

In attempts to study the functional role of noncartilage CSPs in human tumor systems, Mab 9.2.27 was found to block early events of melanoma cell spreading on endothelial basement membranes while only slightly inhibiting cell adhesion. These data suggest that CSPs may play a part in stabilizing cell–substratum interactions in this *in vitro* model for metastatic invasion. Thus, CSPs commonly found in the extracellular matrices of normal cells may function differently in tumor cells lacking a formal structural matrix. An apparent example is the melanoma system described by Bumol *et al.* (1984) that actively synthesizes CSPs but lacks an organized fibronectin matrix.

Certain leukocytes possessing FC receptors are well known to lyse cells coated with specific antibody (Moller, 1965; Perlmann and Perlmann, 1972). Such ADCC is well illustrated by lymphocyte-mediated lysis of antibody-coated tumor cells (Greenberg *et al.*, 1975; Handwerger and Koren, 1976). This classical ADCC reaction has been studied extensively by measuring the lysis of antibody-coated target cells by nonimmune effector cells not previously exposed to antibody. Interactions of the FC portion of the antibody molecule with FC receptors on effector cells are prerequisite for this *in vitro* reaction; however, such reactions are easily inhibited by even low levels of aggregated IgG that competes with target-bound antibody for Fc receptors on effector cells (McLennan, 1972; Larson *et al.*, 1973). Thus, "ADCC-like reactions" *in vivo* would certainly be much impaired by the circulating immune complexes so frequently found in the circulations of cancer patients. One way to avoid inhibition of ADCC by immune complexes is to attach specific antibody to effector cells. This *in vitro* "directed ADCC" proved more effective and less sensitive to inhibition by immune complexes and aggregates of nonimmune IgG than the classical ADCC reaction (Jones and Segal, 1980; Simone, 1982).

In this regard, Schulz *et al.* (1983) observed that murine effector cells armed with Mab 9.2.27 (IgG_{2a}) highly specific for melanoma specifically killed these cells *in vitro* by a "directed ADCC" reaction. In this case, polyethylene glycol 20,000 (8%) was used for nonspecifically enhancing binding of antibody to effector cells. Thus, effector cells were "armed" as first described by Jones and Segal (1980); however, Schulz *et al.* (1983) used fetal calf serum rather than phthalate oils as a separation medium, since the phthalate oils were more difficult to remove and were often toxic to the mice *in vivo*. Data from ^{51}Cr release assays indicated that the directed ADCC was at least twice as effective as the classical ADCC reaction in inducing specific tumor cell killing. In fact, only the directed ADCC reaction clearly exceeded

background; it showed statistically significant reactivity ($p < 0.001$) compared to that of natural killer (NK) cells (Schulz *et al.*, 1983).

2. *In Vivo Reactions in Animal Model Systems*

In initial experiments performed by Koprowski *et al.* (1978), Mab to a melanoma-associated antigen inhibited the growth of human melanoma tumors in athymic, nude mice. Specifically, when such mice received subcutaneously 2×10^6 hybridoma cells secreting an anti-melanoma antibody 2 days prior to receiving a tumor challenge of 1×10^7 cells, the tumor did not establish itself. In fact, histological examination showed only a few inflammatory cells around the area of the necrotizing tumor in mice treated with the Mab. This was in contrast to nude mice receiving control P3 myeloma cells, in which case the injected tumor grew progressively and killed the animals after 36 days.

Herlyn *et al.* (1979, 1980) observed that hybridoma-derived monoclonal anti-colorectal carcinoma Mabs suppressed the growth of colorectal carcinomas in nude mice. The volume of these tumors was lower and the latent period was longer in antibody-treated than in control animals. This was evident when they inoculated 6- to 8-week-old nude mice (BALB/c) under the skin of the neck with 10^7 tumor cells followed within 1 hour by approximately 400 μg of specific antibody injected intraperitoneally. In the first set of experiments, antibody was administered daily for 18 days, and a second set of animals received an additional 4 weekly doses during the 52-day observation period. In yet another experiment, Herlyn *et al.* (1979, 1980) implanted Alzet osmotic minipumps containing a total of 170 μl undiluted ascitic fluid to infuse 1 μl per hour containing 6 μg of specific antibody.

In an attempt to assess specificity as well as the amount of Mab bound *in vivo* to tumors, kidney, and lung cells, the nude mice were injected subcutaneously with 3×10^7 tumor cells and then received, 10 days later, three daily injections each of ascitic fluid containing either Mab to colorectal carcinoma or, alternatively, supernatant of the parent myeloma line used for hybridoma fusion. After 24 hours, binding activity in colorectal carcinoma derived eluates was fourfold higher than in eluates from lungs and kidneys of the injected mice. Eluates of mice injected with melanoma tumor cells as a specificity control were fourfold lower in binding activity (Herlyn *et al.*, 1980). The Mab also bound to cells of three colorectal carcinomas removed from patients but not to adjacent intestinal mucosa from one of these patients (Sears *et al.*, 1982a). Herlyn *et al.* (1980) are careful to point

out that, although the Mab evoked ADCC *in vitro*, they are not certain whether mouse Mab can indeed evoke ADCC with human rather than mouse killer cells. In addition, even though the antibody adsorbed to xenogeneic tumor grafts in nude mice, they stress that it may not recognize a syngeneic tumor in a human host. The precise mechanism of action by which the mouse Mab suppresses growth of human colorectal carcinoma in nude mice is not entirely clear; however, involvement of either complement-dependent cytotoxicity or mature T cells can be ruled out. This is the case since the antibody fails to fix complement *in vitro* and because athymic, nude mice lack mature T cells.

As far as involvement of complement is concerned, Herlyn and Koprowski (1981) observed that four hybridoma cell lines which produce colon carcinoma-specific Mabs of IgM isotype mediated specific complement-dependent cytotoxicity against human colon carcinoma cells but definitely failed to inhibit the growth of colon carcinoma tumors in nude mice. Similarly, only Mab of the IgG_{2a} isotype was effective in suppressing the growth of human tumors in athymic (*nu/nu*) mice in their experiments.

Herlyn *et al.* (1982) later pointed out that of 23 different Mabs tested, only those with the IgG_{2a} isotype inhibited growth of human tumors in nude mice. This probably rules out the possible role of T cells as mediators of the Mab effect in nude mice, since it is most unlikely that such cells would function as effectors in these athymic animals. Although killer cell activity is high in adult nude mice, their role as effectors was thought to be limited, because Mabs effectively inhibited tumor growth in 10- to 12-day-old nude mice whose spleens showed no killer cell activity. Any role of complement in Mab-induced tumor cell suppression was also held to be negligible. Although none of the IgG_{2a} Mabs tested exhibited complement-dependent cytotoxicity with tumor cells in culture, these antibodies frequently bound complement from nude mouse. To investigate the possible role of complement in tumor suppression by IgG_{2a}-type Mabs, adult nude mice were implanted with colorectal carcinoma cells and injected with Mab 17-1A. Suppression of tumor growth was equally effective irrespective whether all detectable complement component C3 was eliminated after administration of cobra venom factor. The results of Herlyn and Koprowski's (1982) experiments made it very clear that, at least in their system, macrophages bearing Fc receptors are most important "effector cells," since either elimination of the Fc fragment from the Mab or inactivation of macrophages by injection of silica particles (1.5 μm) each independently and totally abrogated the effec-

tiveness of Mab in suppressing the growth of human tumor cells in nude mice.

In a human melanoma xenotransplant model, Bumol *et al.* (1983) demonstrated suppression of tumor growth in nude mice evoked by Mab 9.2.27 directed to a CSP preferentially expressed by human melanoma cells. This suppression equaled that achieved by a diphtheria toxin A chain (DTA) conjugate with this same antibody. These investigators also demonstrated in this immunotherapy model that low doses of antibody (40 μg) administered intraperitoneally at 3-day intervals could suppress by 60% the growth of tumors (0.5–1 cm^3) fully established in the nude mice prior to any administration of Mab. This was achieved by injecting relatively large doses of tumor cells (1.5×10^8 cells) and allowing the tumor to establish palpable lesions during a period of 3 days. The Mab and its toxin conjugate bound specifically to melanoma cells *in vitro* but only the conjugate consistently inhibited protein synthesis *in vitro* in these tumor cells. Yet, the antibody by itself as well as its DTA conjugate both suppressed tumor growth *in vivo* to approximately the same extent. Bumol *et al.* (1983) explain that their data do not rule out a possible immunological effect of 9.2.27 IgG_{2a} resulting in suppressed tumor growth. They believe that the biological properties of CSP antigens at the melanoma cell surface, potentially masked by binding of 9.2.27, and concurrent immunological effects of 9.2.27 IgG_{2a} with the host immune system, both act in concert to suppress melanoma tumor growth *in vivo*. However, it should be emphasized that despite 60% suppression of tumor growth in these experiments, residual melanoma cells later formed tumors that ultimately killed the nude mice. The data demonstrate that, under ideal conditions, Mab per se may temporarily prevent tumor establishment, but that this regimen will not permanently inhibit the growth of established tumors.

It was against this background of events and the encouraging results obtained *in vitro* with murine effector cell–antibody conjugates in the directed ADCC that related experiments were initiated *in vivo* (Schulz *et al.*, 1983). Some encouraging observations were made initially when nude mice implanted subcutaneously with human melanoma cells (7.5×10^6) received, 1 day later, several intravenous injections of 2×10^6 effector cells, i.e., mononuclear cells from normal BALB/c mice, "armed" with 50 μg of Mab 9.2.27 IgG. Only in this group of animals did necrotic lesions remain that were "biologically dead" once the injection of conjugates stopped after 12 days. Another group of animals that received an equal number of injections of 40 μg 9.2.27 IgG showed 60% suppression of tumor growth similar to that

previously observed under these conditions by Bumol *et al.* (1983). Again, as in these previous experiments, the effect was transient. This was also true when effector cells either alone, or treated with 8% polyethylene glycol, were administered to several groups of animals. In other words, at day 32, when the experiments were terminated, tumors were growing rapidly in all treatment groups, with the notable exception of those animals that received the effector cells "armed" with Mab 9.2.27 (Schulz *et al.*, 1983).

Nevertheless, the results from these experiments did not confirm that effector cells "armed" with a specific antitumor Mab effectively inhibited the growth of established tumors. This became fact in another set of experiments reported by Schulz *et al.* (1985). Simultaneous injection of Mab 9.2.27 and cell populations with NK activity caused the eradication of established melanoma tumors (mean tumor volume 90 mm^3) in nude mice. Thus, BALB/c nude mice with 2-week-old melanoma tumors received one of the following as a single intravenous injection: 400 μg 9.2.27 IgG; mononuclear BALB/c splenocytes (2×10^7); 2×10^7 mononuclear splenocytes together with 400 μg 9.2.27 IgG. A group of tumor-bearing control mice received no injections. Seven of 10 animals injected simultaneously with splenocytes and Mab 9.2.27 were tumor-free 4 weeks after injection. All other animals treated with either antibody or splenocytes alone exhibited large tumors with the exception of one animal in the group that received only splenocytes. Mice that received only Mab 9.2.27 showed a mean tumor volume that was almost as large as those of control animals, whereas tumors of mice given only effector cells were 50% smaller. Most interestingly, the mean tumor volume in mice that received Mab 9.2.27 together with effector cells was less than 10% of that in controls and only in these animals was tumor destruction permanent (Schulz *et al.*, 1985).

Although mature T cells apparently did not play a major role in the latter tumor cell destruction, several observations strongly suggest that cells with NK activity are the effectors in this process. In this regard, splenocytes obtained from NK-deficient beige C57BL/6 mice were incapable of inducing tumor regression. Specifically, C57BL/6 nude mice with established human melanoma tumors were injected with 9.2.27 IgG, alone or together with splenocytes from NK-deficient C57BL/6 mice that carried the homozygous beige mutation. As the results illustrated in Fig. 3 clearly indicate, none of 10 mice that received normal C57BL/6 splenocytes and 9.2.27 IgG showed any sign of a tumor 4 weeks after injection. In contrast, eight of nine mice that received splenocytes from C57BL/6 *bg/bg* mice had large tumors.

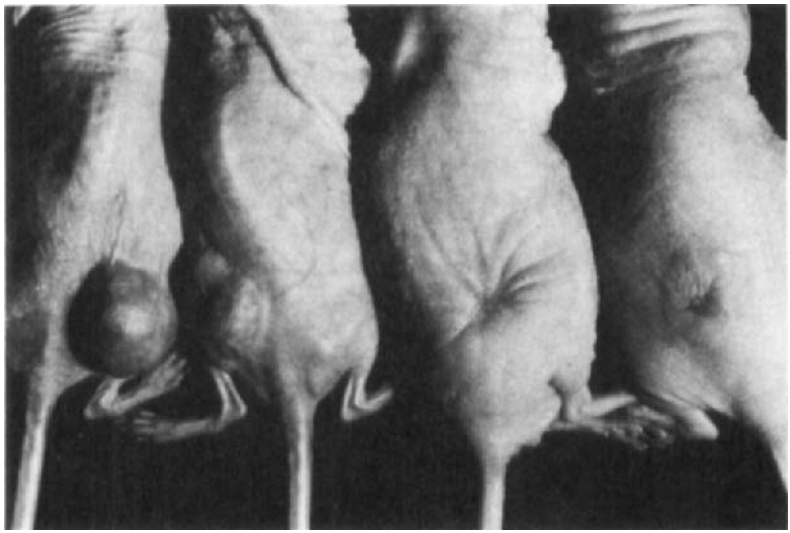

FIG. 3. Growth of human melanoma tumors in nude mice: Effect of Mab 9.2.27 and murine effector cells. The two mice on the left represent the first group of eight C57BL/6 nude mice injected once intravenously with Mab 9.2.27 (400 μg) and mononuclear splenocytes (2×10^7) obtained from C57BL/6 beige mice, *deficient in NK cells*, 14 days after 2×10^7 M21 melanoma cells were injected subcutaneously into these animals. The two mice on the right, apparently devoid of tumor, represent a second group of C57BL/6 nude mice treated in all respects identically to the first group, except that the effector splenocytes injected into them with Mab 9.2.27 were obtained from normal C57BL/6 mice. Sizes of tumors at start of treatment were the same for both groups, i.e., 90–100 mm^3. Photographs were taken 28 days after antibody and effector cell injection, i.e., 42 days after tumor cell inoculation.

Since beige mice have low NK activity, these data strongly suggest that NK cells play a key role in the eradication of established human melanoma tumors in the nude mouse model system (Schulz *et al.*, 1985).

NK cells may not be the only effector cells to be considered in Mab-mediated melanoma tumor eradication of nude mice. Steplewski *et al.* (1983) reported that macrophages isolated from melanoma and colorectal carcinoma patients as well as cultured monocytes expressed Fc receptors that cross-reacted strongly with murine Mabs of the IgG_{2a} isotype, and macrophages in the presence of such antibodies mediated the killing of tumor cells *in vitro*. These data further suggest that Mabs of the IgG_{2a} isotype may be useful in immunotherapy for human cancer.

As far as specific targeting of chemotherapeutic drugs with Mab is concerned, a recent report by Kanellos *et al.* (1985) demonstrated the

effectiveness of covalent conjugates of Mabs and methotrexate (MTX) in producing regression of human colorectal tumors (HT-29) established in nude mice. Up to 50% tumor regression was achieved when six mice, each bearing tumors of 0.3 cm in diameter, were injected intraperitoneally with nine doses of conjugate averaging 1 μg MTX and 64 μg antibody per dose over a period of 38 days. The tumors did not regress when either Mab alone or an unconjugated mixture of antibody and MTX was administered at the same dose schedule to two additional groups of mice bearing human colorectal tumors of similar size. The MTX-to-antibody ratio averaged from 8 to 13 molecules of MTX coupled via the carbodiimide reaction to each molecule of Mab 250-30.6 (IgG_{2b}). Although there was still 50% tumor regression after 38 days in the conjugate-treated animals vs control animals, tumors had reached at least triple the original size. More importantly, tumor size increased sharply in the conjugate-treated animals during the last 8 days of the experiment, suggesting that the effects of the Mab–MTX conjugate on tumor growth become less effective with time.

The observations by Varki *et al.* (1985) are somewhat more encouraging and indicate a more lasting effect of Mab–MTX conjugates in nude mice bearing xenografts of human lung adenocarcinoma. Specifically, their tumor growth curve continually decreased and if anything, levelled off with increasing time. Admittedly, these studies were performed in a different tumor system and with a different Mab, i.e., KS1/4 (IgG_{2a}) of a different isotype than those reported by Kanellos *et al.* (1985). In addition, although the coupling method and MTX-to-Mab ratio were the same as those of Kanellos' group, Varki *et al.* (1985) injected fourfold more antibody and MTX. This may explain in part the greater effectiveness of their Mab–MTX conjugate in controlled tumor growth.

Baldwin *et al.* (1985) evaluated the efficacy of a murine Mab (791 T/36) which could specifically kill osteogenic sarcoma cells *in vitro* and *in vivo* once it had been covalently conjugated with MTX. Conjugates containing from two to three molecules of MTX per molecule of Mab maintained their binding reactivity with osteogenic sarcoma cells to varying degrees, i.e., from 36 to 75%. Direct binding of more than three molecules of MTX per molecule of Mab IgG resulted in almost complete loss of binding activity. When the MTX substitution was increased up to 38 molecules MTX per molecule of Mab IgG through the use of a human serum albumin carrier, antibody-binding activity decreased to as low as 28–36%; however, the conjugates became more cytotoxic *in vitro* and were in this regard comparable to the free drug. *In vivo* tests were done in osteogenic sarcoma-bearing mice that were

immunodeprived by thymectomy and whole body irradiation. When mice were treated 4 days after tumor inoculation with a total dose of 17.5 mg/kg body weight of free or conjugated MTX, there was considerable toxicity with free MTX resulting in 6 of 10 mice dying and tumors developing in 3 of the 4 survivors. None of the mice treated with the Mab–MTX conjugate died and only 4 of 10 developed tumor. Rather surprising was that relatively large tumors (6.5 mm mean tumor diameter) developed in 9 of 10 mice during the entire time, i.e., 25 days, they received 7 injections of Mab–MTX conjugate. However, curiously enough, 4 days after the last injection, only 3 of 10 animals had any tumors and these averaged less than half the original tumor size, i.e., 3 mm diameter. In another trial, where mice were injected twice for 5 weeks with either MTX alone or Mab–MTX conjugate, the latter appeared more effective in reducing tumor weight than free MTX. Specifically, the dose of MTX required to produce 50% inhibition of tumor growth was 17 mg/kg body weight for the Mab–MTX conjugate but 33 μg/kg for the free drug.

III. Ganglioside Antigens

Several key studies are summarized here that deal with the development and characterization of murine Mabs to tumor-associated ganglioside antigens. The focus is mainly on those antigens that have been examined in some depth as to their potential functional roles as tumor markers. Consequently, this article should not be considered a complete review of the field of glycolipid antigens. A number of comprehensive reviews have been published recently that deal with defined glycolipid antigens (Hakomori and Kannagi, 1983; Hakomori, 1984, 1985; Springer, 1984; Feizi, 1985; Cheresh, 1985).

These reviews describe a series of carbohydrates that serve as potential antigens on the surface of cells when attached to either a protein or lipid backbone. The specificity and function of these carbohydrate determinants can now be addressed with the use of Mabs. Numerous studies have characterized Mabs directed to the carbohydrate portion of glycolipids, particularly those containing one or more sialic acid residues, i.e., gangliosides (Magnani *et al.*, 1980; Koprowski *et al.*, 1981; Kemshead *et al.*, 1981; Cahan *et al.*, 1982; Nudelman *et al.*, 1982; Pukel *et al.*, 1982; Hakomori and Kannagi, 1983; Springer, 1984; Hakomori, 1984, 1985; Cheresh *et al.*, 1984a–c; Alejandro *et al.*, 1984; Schulz *et al.*, 1984; Hiabayashi *et al.*, 1985; Feizi, 1985; Cheresh, 1985). Figure 4 is a schematic representation of ganglioside structures designated according to the nomenclature of Sven-

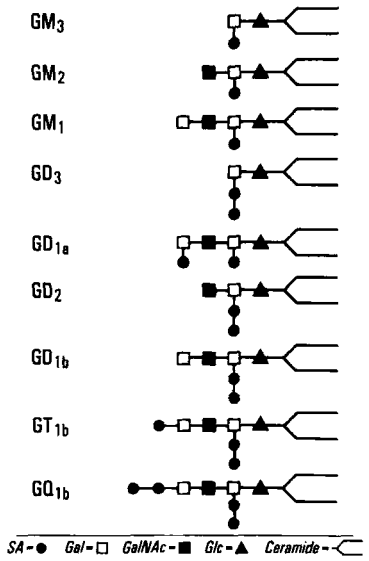

FIG. 4. Schematic representations of ganglioside structures according to the nomenclature of Svennerholm (1963). SA, Sialic acid; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; ceramide, lipid portion of the ganglioside molecule.

nerholm (1963). Recent technological advances have now made it possible to characterize Mabs directed against glycolipids (Magnani *et al.*, 1980; Cheresh *et al.*, 1984a) mainly by immunostaining individual glycolipid determinants separated on a thin layer chromatography plate. This technology compares to indirect immunoprecipitation and/or Western blot analysis, which have facilitated the characterization of Mabs directed to numerous protein and glycoprotein antigens.

One distinct advantage of Mabs directed to carbohydrate antigens is their potential use to establish a structure–function relationship for these determinants on the tumor cell surface. This is possible primarily because Mabs can recognize a carbohydrate determinant with known sugar composition and anomeric linkages. Thus, by using Mabs to defined oligosaccharide structures on the tumor cell surface, one can pose questions regarding the functional properties of these structures as they relate to the malignant or metastatic phenotype. This is much more difficult to do with Mabs directed to protein or glycoprotein antigens whose epitopes are in most cases structurally ill defined, since they depend on unpredictable conformation and three-dimensional structure, and their complete primary amino acid sequence frequently is not available.

A. Proposed Functional Properties of Cell-Associated Gangliosides

The actual function of glycolipids on the cell surface has not been completely delineated, although several researchers using biochemical and immunochemical approaches have proposed that glycolipids (particularly gangliosides) are involved in cell–substratum interactions (Kleinman *et al.*, 1979; Yamada *et al.*, 1981, 1983; Perkins *et al.*, 1982; Spiegel *et al.*, 1984; Okada *et al.*, 1984; Cheresh *et al.*, 1984b, 1986; Cheresh and Klier, 1986) and thus potentially serve as cell surface receptors for the adhesive protein fibronectin (Kleinman *et al.*, 1979; Yamada *et al.*, 1981; Perkins *et al.*, 1982). Gangliosides have also been implicated in some receptor functions, because they specifically bind tetanus and cholera toxins (van Heyningen, 1974) and interferon (Besancon and Ankel, 1974). Consequently, they have been designated as potential receptors for thyroid-stimulating hormone (Mullin *et al.*, 1976) and macrophage inhibition factor (Lui *et al.*, 1982). The α subunit of cholera toxin is known to bind and cross-link five GM1 molecules on the surfaces of rat thymocytes. Thereby, it actually evokes the mitogenic stimulation of such cells (Spiegel *et al.*, 1985), suggesting that gangliosides expressed on the surface of a cell can partake in the potentiation of a transmembrane signal resulting in lymphocyte activation. In addition, recent evidence suggests that cell surface gangliosides can modulate glycoprotein receptor function (Bremer *et al.*, 1984; Berry-Kravis and Dawson, 1985). Thus, in the presence of the disialoganglioside GD3, the serotonin receptor on neural cells shows an increased affinity for serotonin (Berry-Kravis and Dawson, 1985). In addition, the receptor for platelet-derived growth factor can be functionally modulated by gangliosides (Bremer *et al.*, 1984). The addition of an exogenous calcium/ganglioside complex to rat brain membranes resulted in the direct phosphorylation of five proteins and the inhibition of three others (Goldenring *et al.*, 1985), further suggesting that gangliosides on the cell surface are in some way involved in potentiating a transmembrane signaling event. Specific gangliosides have also been implicated as host cell receptors for viruses (Markwell *et al.*, 1981; Suzuki *et al.*, 1985).

In this regard, Sendai virus was shown to bind to gangliosides with structures similar to GD1a, GT1b, and GQ1b, all of which contain at least one sialic acid attached to a terminal galactose residue (Markwell *et al.*, 1981). However, gangliosides lacking this moiety (i.e., GM1 and GD1b) are incapable of interacting with the virus. In addition, recent evidence indicates that the monosialoganglioside GM3 serves as a

specific receptor for influenza A virus, thereby allowing for the adsorption–fusion process of viral infection (Suzuki *et al.*, 1985). In this study, evidence was presented that the critical determinant involved a sialic acid moiety connected via a 2–3 linkage to an external galactose present on a ganglioside. Thus, depending on the particular oligosaccharide composition and structure, gangliosides have a wide range of potential biological functions and are therefore useful to study as cell surface antigens.

The fact that tumor cells express altered carbohydrates on both glycolipids and glycoproteins suggests that Mabs directed to such determinants may help to elucidate some of the structural changes associated with malignant transformation. This, in turn, may also lead to an understanding of certain genetic abnormalities or modifications within the tumor cell, since the expression of carbohydrate antigens is under the direct control of a variety of glycosyltransferases. In a recent study, transfection of rat cells with the transforming gene of human adenovirus type 12 DNA and its transcriptional subunits induced the synthesis and expression of the disialoganglioside GD3 (Nakakuma *et al.*, 1984). The synthesis of this ganglioside was subsequently shown to result from activation of a highly specific sialyltransferase, i.e., GD3 synthetase (Nagai *et al.*, 1984). The expression of glycolipids on cell surfaces can also be under epigenetic control and thus vary according to cell cycle (Gahmberg and Hakomori, 1974), density (Hakomori, 1970), and cell contact (Yogeeswaran and Hakomori, 1975); in some cases their expression is directly linked to a particular stage in differentiation (Hakomori, 1981; Hakomori and Kannagi, 1983; Feizi, 1985), suggesting that their appearance on the tumor cell surface may be "oncofetal" in nature. Based on this hypothesis and the fact that these molecules are readily shed from the plasma membranes of tumor cells (Hakomori and Kannagi, 1983; Schulz *et al.*, 1984; Cheresh, 1985), glycolipid antigens proved to be useful markers for the immunodiagnosis of certain tumors (Portoukalian *et al.*, 1979; Hakomori and Kannagi, 1983; Schulz *et al.*, 1984; Cheresh, 1985).

Recent studies in mice (Cheresh *et al.*, 1985) and in man (Houghton *et al.*, 1985) indicate that Mabs directed to certain glycolipid tumor-associated antigens may be particularly useful in the immunotherapeutic treatment of cancer patients. Results from our laboratory show that human melanoma cell lines derived from a patient's metastatic lesions have a marked increase in the expression of the disialogangliosides GD2 and GD3 compared to cell lines derived from primary melanoma lesions of the same patient (Rosenberg *et al.*, 1986). The increased synthesis and expression of GD3 in these cells corre-

lates directly with the increased activity of a specific sialyltransferase that converts the monosialyated ganglioside GM3 to GD3 (Rosenberg *et al.*, 1986). Based on these studies, Mabs directed to glycolipid antigens, particularly gangliosides, can potentially serve as useful reagents for immunodiagnosis, immunotherapy, and biological characterization of malignant cells.

Most of the information regarding the functional role of glycolipids on the cell surface has come from studies involving their addition to cultured cells with the assumption that these glycolipids appropriately embed in the lipid bilayer. However, this work has been greatly facilitated by the use of Mabs directed to the carbohydrate portion of the molecule naturally exposed on the cell surface. Moreover, with these Mabs, much can be learned about the structural properties of glycolipids and the role they play as antigens on the surfaces of tumor cells. Glycolipids in a pure form are relatively nonimmunogenic; however, upon appropriate antigen presentation one can generate highly specific Mabs to the carbohydrate portion of these molecules. In fact, Mabs directed to glycolipid antigenic determinants can have a similar degree of precision and specificity as that of Mabs to antigenic epitopes on proteins. As with any antigen, the density of a given glycolipid on the cell surface plays a key role in its antigenicity and potential immunogenicity. Other factors have been implicated in cell surface glycolipid antigen expression, including the chain length and ceramide composition (lipid backbone) as well as coexisting glycolipids (Kannagi *et al.*, 1983). Although the antigenic portion of the glycolipid involves its oligosaccharide moiety, the ceramide composition, which may effect glycolipid organization in membranes, and coexisting glycolipids may also play significant roles in the antigenicity and/or immunogenicity of glycolipid on the cell surface.

B. Structural and Functional Properties of Ganglioside Antigens Associated with Tumor Cells

1. Neuroblastoma

Neuroblastoma, a neoplasm of the peripheral autonomic nervous system, is the second most common solid tumor in children. The majority of children initially diagnosed with neuroblastoma already have advanced disease (stages III and IV), for which treatment protocols have proved largely unsuccessful (Seeger *et al.*, 1982). In fact, once detected, many neuroblastomas in children are quite difficult to distinguish histopathologically from other round cell tumors such as lymphoblastic lymphoma, leukemia, rhabdomyosarcoma, or Ewing's sar-

coma (Raney *et al.*, 1976; Reynolds *et al.*, 1981). Therefore, Mabs to antigens on the surfaces of neuroblastomas have considerable immunodiagnostic and immunotherapeutic potential. Eisenbarth *et al.* (1979) were among the first to describe such a reagent—a murine Mab (A2B5) reactive with human neuroblastoma cells and a plasma membrane antigen on neurons. The antigen defined by Mab A2B5 was present in a GQ (containing four sialic acids) ganglioside fraction from bovine brain. Indirect immunofluorescence studies demonstrated that the ganglioside recognized by Mab A2B5 was associated with the plasma membrane of most neuron cell bodies but was not detected on axons or dendrites, Melur cells, or retina pigment cells. This Mab was later shown to react with the GQ1b ganglioside on neuroblastoma cells and was used to discriminate between hemapoietic cells and infiltrating neuroblastoma cells in bone marrow (Kemshead *et al.*, 1981), indicating its utility in immunodiagnosis. GQ1b on the surface of human neuroblastoma cells has been implicated in the ability of such cells to establish neurite outgrowths and has been suggested to enhance cell proliferation (Tsuji *et al.*, 1983).

In our own laboratory, Mabs were generated against intact human neuroblastoma cells (LAN-1), which are known to express an abundant level of the disialoganglioside GD2. After screening the hybridoma supernatants with a solid phase lipid ELISA, three anti-glycolipid IgMs and two IgG_3 antibodies were isolated and later shown to react specifically with the oligosaccharide portion of GD2 (Cheresh *et al.*, 1984b). These Mabs did not react with the carbohydrate portion of glycoproteins, since they failed to immunoprecipitate protein or recognize one by Western blot analysis (Cheresh and Klier, 1986). We then used these reagents to detect this antigen on cultured human tumor cell lines as well as on malignant and normal tissues. Results from ELISA of the cultured cells demonstrated that GD2 was heavily expressed on lines derived from human neuroblastoma, melanoma, glioma, as well as small cell cancer of the lung (Schulz *et al.*, 1984). Indirect immunoperoxidase assays of either frozen sections or formalin-fixed tissues also revealed that GD2 was preferentially expressed on tissues derived from the neuroectoderm (Schulz *et al.*, 1984), including normal adult and fetal brain. Mab 126, (IgM) directed against this antigen, was then used to develop an extremely sensitive serum assay for GD2 in patients with active neuroblastoma. The need for a reliable serum immunodiagnostic/prognostic assay for the detection of this deadly childhood tumor is most apparent when one considers the difficult in obtaining biopsy material.

The key to the development of this assay was a brief extraction of the serum sample with chloroform/methanol (2/1) before its incuba-

tion with the antibody. Based on a competitive inhibition curve using authentic GD2 as a standard, it was possible to detect the actual GD2 level in 250 μl of serum (Schulz *et al.*, 1984). This assay demonstrated that sera from 21 of 23 children with various stages (I–IVs) of active neuroblastoma contained significantly elevated levels of GD2 ranging from 50 to 4300 ng/ml as compared with 16 of 16 normal children having levels ranging from 1 to 30 ng/ml ($p < 0.001$). These results indicate the potential of such a prognostic test for neuroblastoma, since the level of GD2 directly correlates with tumor burden in a number of patients after surgery or chemotherapy (Schulz *et al.*, 1984).

Additional murine anti-GD2 Mabs have since been described by Saito *et al.* (1985). In a follow-up report (Saarinen *et al.*, 1985), one of these Mabs (3G6), an IgM, used with human complement, purged autologous bone marrow of neuroblastoma cells *in vitro*. This complement-mediated cytotoxicity technique killed 99.9–100% of several human neuroblastoma cell lines, yet normal marrow precursor cells were not detectably damaged. The results from this study were comparable to those obtained using a 12-Mab panel bonded to magnetic beads, previously providing the best reported results (Treleaven *et al.*, 1984). This anti-GD2 Mab is now being used clinically to treat autologous bone marrow of patients with stages III and IV neuroblastoma. The results should help to clarify issues concerning engraftment and long-term remission and survival (Saarinen *et al.*, 1985).

2. *Melanoma*

Human melanoma, similar to neuroblastoma, is derived from the neuroectoderm and rich in ganglioside content. A number of reports have described murine Mabs directed to the disialoganglioside GD3 which is similar in structure to GD2 but lacks a terminal *N*-acetylgalactosamine. Early studies described Mabs R-24 (IgG_3) (Pukel *et al.*, 1982) and 4.2 (IgM) (Nudelman *et al.*, 1982), both of which were reported to recognize GD3 specifically on the surfaces of human melanoma cells. Mab 4.2 was shown to react with melanoma-associated GD3 by enzymatic degradation and mass spectrometry, which revealed a carbohydrate moiety identical to brain GD3, yet is ceramide manifested a predominance of longer chain fatty acids than brain GD3. Nudelman *et al.* (1982) suggested that, because GD3 in melanoma contained a longer ceramide, its organization in the membrane probably provoked far greater antigenicity than that of GD3 in normal cell membranes. Thus, GD3 in normal cells may be cryptic, whereas in the melanoma membrane it is highly exposed. A similar situation has been described for the monosialylated GM3 in baby hamster kid-

ney cells or globoside in NIL cells; this GM3 became exposed at the cell surface when these cells were transformed with polyoma virus (Hakomori *et al.*, 1968).

Our laboratory also produced an anti-GD3 Mab of IgG_3 isotype (Mab MB3.6) made against human melanoma cells (Cheresh *et al.*, 1984b). Using this Mab as well as Mab 126 directed to GD2, we could localize each of the gangliosides in focal adhesion plaques and on the surfaces of human melanoma cells (Cheresh *et al.*, 1984b). As illustrated in Fig. 5A, human melanoma cells stained with Mab 126 and examined by indirect immunofluorescence exhibited intense surface fluorescence as well as specific staining of membrane associated microprocesses. These structures emanate from the plasma membrane and, in some cases, appear to make contact with the substrate to which these cells are attached. This contrasts with the punctate staining pattern of Mab 9.2.27 (Fig. 5B), which detects a glycoprotein antigen, i.e., a CSP, on these cells (Bumol and Reisfeld, 1982; Bumol *et al.*, 1984; Harper *et al.*, 1984) that appears to localize on the surface and in the extracellular matrix but not in focal adhesion plaques. This evidence further supports the suggestions of others (Yamada *et al.*, 1981, 1983; Spiegel *et al.*, 1985; Okada *et al.*, 1984; Kleinman *et al.*, 1979; Perkins *et al.*, 1982) that cell surface-expressed gangliosides may be involved in cell–substratum interactions. The melanoma-associated adhesion plaques (generated by treating melanoma cells attached to glass coverslips with EDTA) do not represent indiscriminant membrane fragments, because neither MPG nor class I histocompatibility antigens are detected by their respective antibodies (Cheresh *et al.*, 1984b). Further studies did established that GD2 and GD3 are involved in a generalized mechanism of melanoma cell attachment (Cheresh *et al.*, 1986). Anti-GD2 as well as anti-GD3 were capable of inhibiting the attachment of human melanoma and neuroblastoma cells to a variety of immobilized extracellular matrix components including: fibronectin, vitronectin, collagen, and laminin (Cheresh *et al.*, 1986). The role of gangliosides in melanoma cell attachment to a more physiological substrate has also been examined. As shown in Fig. 6, pretreatment of M21 melanoma cells with anti-GD2 antibody resulted in 92% inhibition of attachment to a matrix of bovine endothelial cells laid down on tissue culture plastic. Kinetic analysis of this inhibition suggested that these gangliosides were involved in the initial phases of the cell attachment process, since inhibition was the most significant within the first 5–10 minutes of cell attachment (Cheresh *et al.*, 1986). Moreover, these Mabs induced cell rounding and loss of attachment by cells preadhered and spread on a fibronectin substrate. This confirmed an earlier report by Dippold *et al.* (1984),

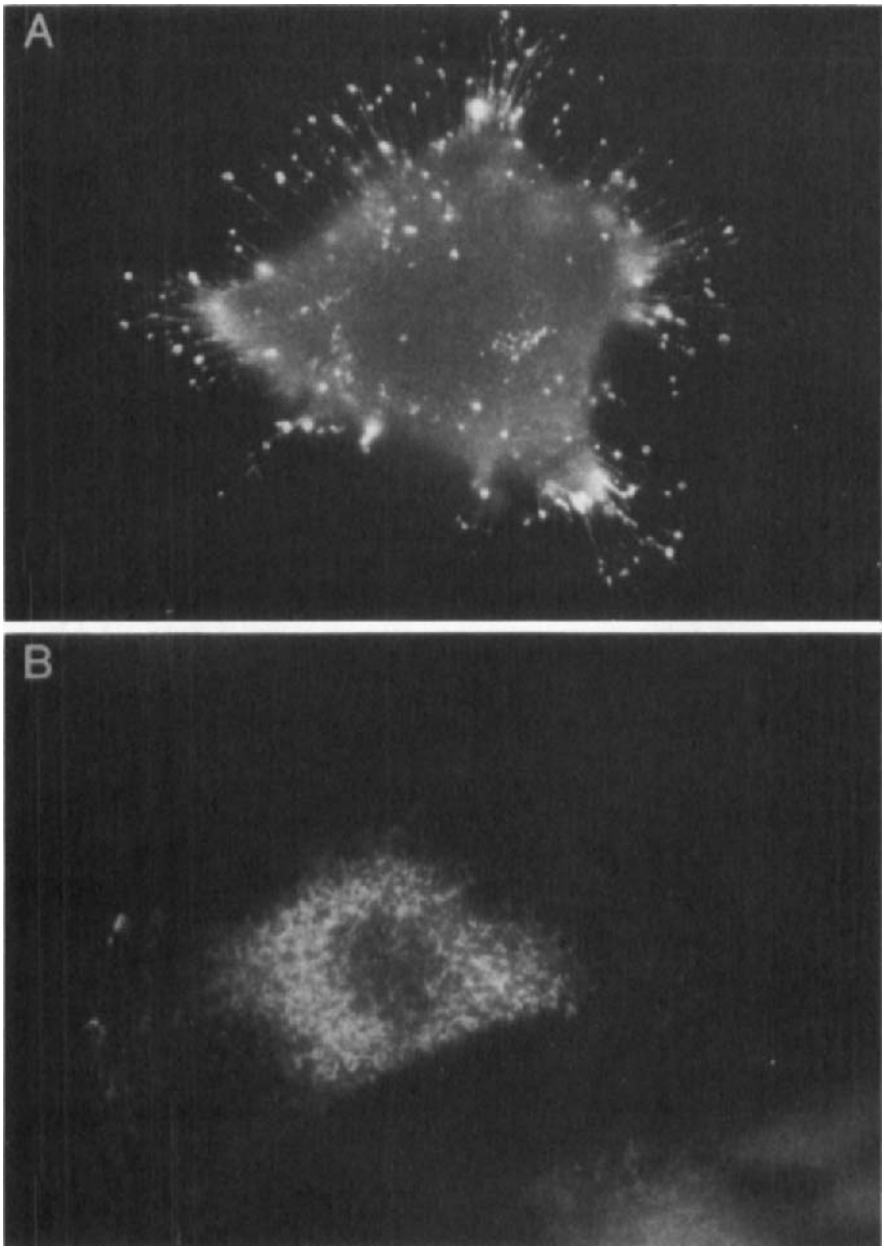

FIG. 5. Immunolocalization of GD2 on the surfaces of human melanoma cells as defined by Mab 126. Human melanoma cells (Melur) were allowed to attach to glass coverslips, were stained with Mabs 126 (A) or 9.2.27 (B) as primary antibodies, and were examined by indirect immunofluorescence.

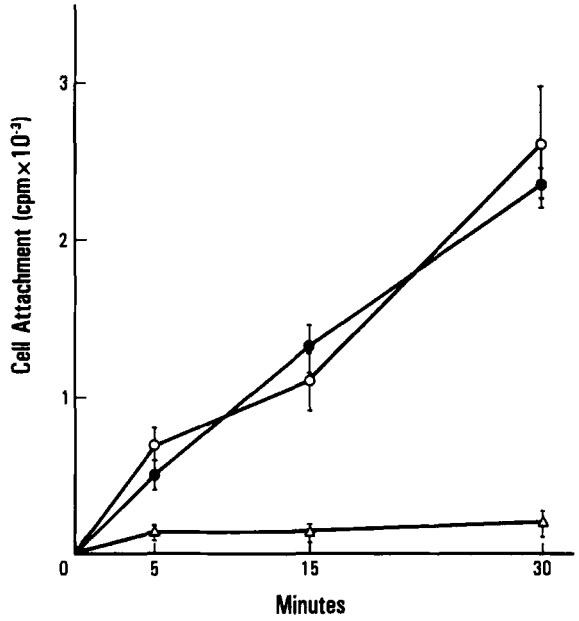

FIG. 6. Anti-GD2 Mab-induced inhibition of M21 human melanoma cell attachment to a bovine endothelial extracellular matrix. Metabolically labeled M21 cells were allowed to attach for various times to microtiter wells coated with the bovine endothelial extracellular matrix as previously described (Cheresh *et al.*, 1986). Before addition to wells, cells were preincubated at 4°C for 1 hour with growth media (○), Mab 9.2.27 (●), or anti-GD_2 Mab 14.18 (△). Each point represents the mean ± SD of four replicates.

that Mab R24 (IgG_3) also directed to GD3 induced similar melanoma cell rounding from tissue culture plastic. In contrast, Mabs directed to other determinants on the cell surface failed to alter the cells' adhesive properties, indicating that the inhibitory effects observed were not simply due to Mab binding to any antigen on the cell surface. Furthermore, an ultrastructural analysis of human melanoma cell attachment using both scanning and transmission immunoelectron microscopy demonstrated that GD2 gangliosides were preferentially expressed on cell membrane-associated microprocesses in direct contact with the substrate upon which the cells were attached (Cheresh and Klier, 1986). In fact, an actual gradient of antigen expression could be observed where ganglioside expression increased on the microprocesses as they neared the substrate. Preliminary evidence obtained in our laboratory now suggests that both GD2 and GD3 can interact directly with melanoma cell surface glycoprotein receptors for fibronectin or vitronectin; thus, this interaction may potentiate the ap-

propriate receptor configuration leading to optimal cell attachment (Cheresh and Claypool, unpublished observations). Mabs directed to gangliosides that are part of a cell attachment-promoting receptor complex on the melanoma cell surface may be expected to interfere with the cell attachment process itself. Therefore, by using Mabs directed to two different ganglioside antigens expressed on the surfaces of human melanoma cells, it was possible to gain insight into their mechanism of attachment to the extracellular matrix, a process that is essential for tumor cells to invade and establish distant metastasis.

A number of other studies have implicated gangliosides expressed on the surfaces of melanoma cells as potentially relevant antigens in man. In this regard, Watanabe *et al.* (1982) described the "AH antigen," which was detected during the initial analysis of the humoral immune response of melanoma patients to autologous melanoma cells. Serum from patient AH reacted with a cell surface antigen expressed by cultured autologous melanoma cells, but not by autologous skin fibroblasts or peripheral blood lymphocytes, and ultimately identified as the GD2 ganglioside. In other studies, peripheral blood lymphocytes from melanoma patients transformed into B lymphoblastoid cells by Epstein–Barr virus were used as an *in vitro* source to generate monospecific IgM antibodies of two kinds, one specifically reactive with GD2 (OFA-I-2) (Cahan *et al.*, 1982) and another directed to the monosialyated version, i.e., GM2 (OFA-I-1) (Tai *et al.*, 1983).

These reports suggest that, depending on the particular oligosaccharide moiety, certain tumor-associated ganglioside antigens can evoke a humoral immune response in man that may aid in the serological detection of some cancers. To date, murine Mabs have probably been more effective for immunohistological diagnosis of human tumor than Mabs of human origin. One reason is that Mabs of murine origin do not have the intrinsic background problems on human tissues that human-derived Mabs do. Using the murine Mab R24 to GD3, Dippold *et al.* (1984) clearly demonstrated the immunohistological localization of this antigen in human malignant melanoma, epithelial tumor, and normal tissues. Schulz *et al.* (1983) also used their murine Mab 126 (IgM) to GD2 for immunohistological studies. However, Cahan *et al.* (1982) were less fortunate because their human monoclonal antibody to GD2 suffered from background problems when tested with human tissues.

Using Mab R24 in conjunction with an indirect immunoperoxidase-staining technique on 175 cryopreserved, unfixed human tissue sections, Houghton *et al.* (1985) established a striking specificity for malignant melanoma. Thus, 21 of 21 of primary and 37 of 37 malignant

melanoma tissue sections contained GD3. Among the numerous normal tissues examined, 9 of 11 nevi were GD3 positive, and melanocytes in the basal layer of the skin stained weakly. The authors concluded that the apparent specificity of Mab R24 for human melanoma tissues was a likely explanation for the lack of serious side effects in patients receiving this Mab in a phase I clinical trial during which several significant tumor regressions were observed. In this clinical trial, melanoma patients receiving Mab R24 developed rapid local inflammatory responses directly at the tumor sites, apparently caused by the deposition of complement components and/or resulting lymphocytic infiltration. Indeed, *in vitro* studies have demonstrated that Mabs directed to GD3 can readily lyse human melanoma target cells in the presence of human complement or peripheral blood mononuclear cells through an ADCC mechanism (Cheresh *et al.*, 1985). Later experimentation showed that large granular lymphocytes displaying NK function were likely involved in this ADCC of human melanoma cell targets (Herberman *et al.*, 1985). One might speculate that a glycolipid antigen such as GD3 serves as a particularly relevant target for Mab therapy of human melanoma. First, because it is copiously expressed on melanomas, and second, because it is embedded in the outer layer of the lipid bilayer, providing a highly sensitive target for cytolytic attack. Furthermore, since anti-ganglioside Mabs can inhibit melanoma cell–substratum interactions (Cheresh *et al.*, 1986), the possibility exists that the rate of tumor cell invasion and subsequent metastasis may be decreased. Quite possibly, a combination of the above mechanisms is involved in the apparent positive response of some melanoma patients to anti-GD3 therapy (Houghton *et al.*, 1985).

Although GD3 is heavily expressed on most human melanoma cells examined, it is also present on other cell types derived from the neuroectoderm. In related research, we have used another Mab (D1.1) that was originally generated against an undifferentiated rat neuronal tumor cell line (B49) as originally described by Levine *et al.* (1984). We now know from our work that this antibody reacts specifically with a 9-O-acetylated form of the disialoganglioside GD3 (Cheresh *et al.*, 1984a,c). The expression of this antigen is even more restricted to human melanoma cells than that of GD3, since in our hands Mab D1.1 did not react with normal melanocytes, fetal or adult brain tissue, or normal skin (Cheresh *et al.*, 1984a, 1985). During chemical characterization of the antigenic epitope defined by Mab D1.1 (Cheresh *et al.*, 1984a), the antigen epitope recognized by this Mab was highly sensitive to mild alkali hydrolysis, i.e., 0.1 *N* NaOH in methanol for 1 hour at 37°C. Furthermore, after base hydrolysis, the D1.1 ganglioside not

only lost reactivity with Mab D1.1 but now comigrated with GD3 on thin layer chromatography (TLC) plates (Cheresh *et al.*, 1984a) and also reacted specifically with the anti-GD3 Mab MB3.6 (Cheresh *et al.*, 1984c). Moreover, in a small amount of this ganglioside isolated by preparative TLC, one of two sialic acids removed by acid hydrolysis comigrated with authentic 9-O-acetylated sialic acid on paper chromatography, suggesting that the antigen recognized by Mab D1.1 may be the as yet undefined alkali-labile acetylated product of disialoganglioside GD3.

To confirm the structure of this ganglioside, we initially used mild periodate oxidation on a ganglioside fraction from human melanoma cells under conditions known to permit oxidation only between the exocyclic (7–8–9) hydroxyl positions of terminal, unsubstituted sialic acid residues (Van Lenten and Ashwell, 1971). Others previously reported that an acetyl group in the 9-position of sialic acid sterically hinders this oxidation, whereas substitutions at the 7- or 4-positions do not (Van Lenten and Ashwell, 1971). Thus, under conditions of controlled periodate oxidation we specifically eliminated the reactivity of Mabs that recognize only nonacetylated forms of either GD3 or GD2, while reactivity with Mab D1.1 remained unchanged (Cheresh *et al.*, 1984c). These data strongly suggest that the *O*-acetyl ester is indeed located on the 9-hydroxyl position of GD3. We also confirmed this finding by chemical means, using the mild acetylating agent, *N*-acetylimidazole, that selectively O-acetylates the 9-position of the methyl ester/methyl glycoside of sialic acid under controlled conditions of molar excess (Haverkamp *et al.*, 1975). The compound created by this procedure now migrated in the appropriate position on TLC and also specifically reacted with Mab D1.1 (Cheresh *et al.*, 1984c). Thurin *et al.* (1985) recently confirmed the structure of 9-*O*-acetylated GD3 by proton NMR and fast-atom bombardment mass spectrometry and described another Mab (ME311) made against human melanoma cell targets that reacted with this antigen. Although they found no evidence of this epitope in mucins or glycoproteins, Thurin *et al.* (1985) did observe some reactivity with normal nevi. This is in contrast to the reactivity of Mab D1.1, which did not react with nevi (Cheresh *et al.*, 1984a). Conceivably, these Mabs react with slightly different epitopes and thus Mab D1.1 may not show the same degree of cross-reactivity as Mab ME 311.

In attempts to delineate the mechanism of O-acetylation of GD3, we examined the possibility that human melanoma cells contain an enzyme responsible for converting GD3 to its O-acetylated form. To this end, lysates were prepared from human melanoma cells and allowed

to react with GD3, which had been previously de-O-acetylated to destroy any preexisting reactivity with Mab D1.1. As previously reported, a specific enzymatic activity mediated the conversion of GD3 to its O-acetylated form (Cheresh *et al.*, 1984c), correlated directly with protein concentration and time, and could be abolished by heating at 80°C for 20 minutes. Thus, the generation of the epitope recognized by Mab D1.1 required two independent events: first, the synthesis of the disialoganglioside GD3, and second, the presence of an O-acetyltransferase capable of acetylating the 9-hydroxyl position of its terminal sialic residue. Although each of these factors may be present individually in other normal tissues and tumors, it is the combination of the two factors in human melanoma cells that results in the generation of an antigen that is apparently tumor specific. The fact that a single acetyl epitope at the 9-position of one of two sialic acids present on GD3, a four-sugar oligosaccharide, can make the difference between two antigenic epitopes clearly illustrates the exquisite specificity obtainable with Mabs directed to carbohydrate antigens. In fact, at least 25 forms of sialic acid have been described to date (Schauer, 1982), allowing for a tremendous diversity of sialic acid-bearing carbohydrate antigens. The specificity of carbohydrate antigens is also demonstrated by the work of Hakomori *et al.* (1983), who recently demonstrated a Mab directed to an *N*-acetylneuraminosyl-α2-6-galactosyl residue on gangliosides of the lacto series as well as on certain glycoproteins. The specificity of this antibody was anomeric linkage dependent, since it did not react with either glycoproteins or gangliosides having an *N*-acetylneuraminosyl-α-2-3 or 2-4-galactosyl residue. This antibody was used to detect small accumulations of gangliosides having this terminal structure in some forms of human cancers. Taken together, the data presented by us and others suggest that Mabs directed to carbohydrate antigens may not only help to define new antigenic structures on tumor cells but also may lead to a better understanding of the aberrant glycosylation patterns thought to exist in malignant tissues.

3. *Colon Carcinoma*

Sialic acid-bearing carbohydrate antigens have also been useful to dissect tumors other than those derived from the neuroectoderm. A recently reported antibody specifically detected *N*-glycolyl-containing gangliosides preferentially expressed on human colon cancer tissues (Higashi *et al.*, 1985) and also known as the Hanganutziu–Deicher (HD) antigen. In this case, four species of antigenic gangliosides were found, indicating that the actual antigenic determinant included

only the *N*-glycolyl sialic acid residue itself. This contrasts with our studies, in which Mab D1.1 recognized only an O-acetylated sialic acid in the context of the entire four-sugar structure of the O-acetylated GD3 ganglioside (Cheresh *et al.*, 1984a,c).

Among the tumor-associated carbohydrate antigens studied to date, the colorectal carcinoma ganglioside determinant defined by Mab 19-9 (Magnani *et al.*, 1981) is probably the most vigorously examined. This Mab was initially shown to be highly specific for cancers originating in the gastrointestinal (GI) tract, particularly colorectal adenocarcinoma, as well as gastric and pancreatic cancer (Koprowski *et al.*, 1979, 1981). Such patients contained the antigen recognized by Mab 19-9, since their sera inhibited the binding of this Mab to colorectal carcinoma tissues extracts, whereas sera from patients with other bowel diseases or from normal donors did this to a far lesser extent. The antigen recognized by Mab 19-9 was originally found in the ganglioside fraction of tumor tissue as well as in meconium (Magnani *et al.*, 1981). The ganglioside was identified as the monosialyated form of the Le blood group A antigen, which was not known to exist on normal tissue. The antigen in sera from colorectal carcinoma patients was later established as a mucin, indicating that the carbohydrate identified by Mab 19-9 could be present on either a protein or lipid backbone (Feizi, 1985). In a comprehensive follow-up study by Ritts *et al.* (1984), clinical evaluation of the 19-9 antigen was performed on 1600 coded sera obtained from donors to the NCI/Mayo Clinic Serum Bank. Results from an immunoradiometric assay indicated that the concentration of 19-9 antigen was elevated in a large fraction of sera (67%) from patients with advanced adenocarcinoma of the GI tract, including those with pancreatic, hepatobiliary, and gastric carcinomas, yet only 4 of 1023 normal sera contained an elevated level. The authors of this study concluded that this assay may have clinical utility as a diagnostic adjunct for adenocarcinoma of the upper GI tract; however, rigorous prospective clinical studies will be necessary for verification. In a multicenter prospective controlled trial, reported by Arends *et al.* (1983) using Mab 19-9 to examine colorectal carcinoma tissue sections, the status of immunoreactivity did not correlate with either localization, stage, histopathological characteristics, or DNA cytometry. Thus, although a study of the 19-9 antigen in tissues may be worthwhile in relation to fundamental aspects of colorectal carcinomas, the significance of its immunohistochemical detection may be somewhat limited for immunodiagnostic or prognostic purposes.

Recently, Steplewski *et al.* (1986) reported the use of Mab 19-9 isotype switch variants to examine the role of the Fc portion of a

murine Mab to potentiate tumor cytolytic activity. Three different Mab isotypes were produced, IgG_1, IgG_{2b}, and IgG_{2a}, and shown to vary in their cytolytic activity *in vitro* and their tumoricidal activity *in vivo*. Only the IgG_{2a} Mabs bound with high affinity to Fc receptors on murine or human macrophages, and this isotype was responsible for efficient antibody-dependent, macrophage-mediated cytotoxicity. In nude mice only the IgG_{2a} variant inhibited growth of human colorectal xenographs indicating that the isotype switch resulted in the conversion of a non-cytolytic into a cytolytic Mab with possible immunotherapeutic application. These extensive studies with the 19-9 antigen suggest that sialic acid bearing carbohydrate tumor-associated antigens are quite useful markers for investigation, both from a clinical as well as a fundamental point of view, and that Mabs directed to such determinants should provide new and very useful reagents for such future efforts.

IV. Phase I Clinical Trials

An early phase I clinical evaluation was performed with IgG_{2a} antimelanoma Mab 225.28S in two patients with massive tumor burden in end stage melanoma (Sobol *et al.*, 1982). The antibody, which recognizes a glycoprotein of M_r 240,000 on the surfaces of human melanoma cells, was given intravenously in 10-mg doses to both patients over a period of 2 hours. Peak serum antibody levels were seen in both patients at the end of infusion, and the half-life of the antibody in serum was 16 hours. No antibody was detectable in their sera after 48 hours; however, neither patient had any sign of circulating tumor antigen preceding therapy. Posttreatment biopsies of 4, 24, and 48 hours revealed Mab binding by fluorescence microscopy. However, no positive therapeutic response, i.e., measurable decrease in tumor lesions, was seen, nor were there clinically adverse or major toxicity effects noted that could be ascribed to this antibody. The investigators proposed several possible explanations to account for the failure of Mab 225.28S to elicit any response. The 10-mg dose may have been too small to cope with the patients' massive tumor burdens, or the antibody may never have left the intravascular compartment to deliver an adequate dose to tumor-binding sites. Finally, *in vivo* modulation of antigen–antibody complexes could not be ruled out. The investigators were unable to say which, if any, of these problems occurred and stressed the positive aspects of their study, i.e., "absence of toxicity following slow i.v. infusion of antibody while pointing out the non-

uniform pharmacokinetics of monoclonal antibody administration" (Sobel *et al.*, 1982).

Sears *et al.* (1982c) reported the results of phase I clinical trials of Mab in the treatment of GI tumors of four patients. The Mab 1083-17-1A (17-1A) used in these studies had previously mediated lysis of colorectal carcinoma cells by human or mouse effector cells and specifically suppressed the growth of human colon carcinoma xenografts in athymic (*nu/nu*) mice (Herlyn *et al.*, 1979). This Mab is directed to a protein antigen of M_r 38,000 which was not shed during tumor cell culture and persisted in the circulation for 2 weeks or longer when more than 15 mg was injected intravenously. Doses ranged from 15 to 200 mg of the purified Mab injected once. Three of these four patients produced human anti-mouse antibody. Patient 1 received 15 mg of Mab 17-1A, which remained in his circulation 48 hours. Patients 2 and 3, who received 180 and 150 mg of 17-1A, maintained human anti-mouse antibodies in their circulations for 110 and 50 days, respectively, showing peak levels 11–14 days after treatment. Patient 4, who received repeated injections of antibody 17-1A over a period of 10 days, had the highest serum levels of human anti-mouse antibody within 24 hours and decreasing linearly from days 11 to 14 after treatment. Human anti-mouse antibodies were first detected 9 days after treatment and increased steadily throughout the next 21 days. The circulating Mab 17-1A of patient 4 also bound to cultured colon carcinoma cells. Patients 1, 2, and 3 had no side effects from Mab 17-1A. Unfortunately, the effect of a single injection on tumor growth could not be measured since surgical intervention was required for all three patients. Only patient 4 could be evaluated in this respect. He received the first 200 mg antibody intravenously, then another 67 mg of antibody the next day. This second injection was a mixture of the patient's own mononuclear cells and the Mab, which had been preincubated for 30 minutes at room temperature before use and was administered via a hepatic artery catheter. This patient complained of some epigastric discomfort and showed transient increases in serum aspartate aminotransferase and lactic dehydrogenase levels. He also underwent a laparotomy of the hepatic metastases and liver ultrasound examination. Inspection of clavicular metastasic biopsies followed. Three weeks after Mab administration, the metastases were much smaller and their echogenic characteristics had changed. Encouragingly enough, patient 4 showed no signs of serum sickness when examined for 2 weeks as an outpatient, and he had normal renal function without proteinuria.

The choice of this patient's own mononuclear cells as a "carrier" of the Mab is an interesting one, based, no doubt, on the observation that purified peripheral blood mononuclear cells exposed to Mab 17-1A effectively destroyed colorectal carcinoma cells in athymic (*nu/nu*) mice (Herlyn *et al.*, 1980).

Mab 9.2.27 directed to a proteoglycan antigen expressed on human melanoma was also tested in limited phase I clinical trials by Oldham and colleagues (1984). Each patient received single doses of Mab 9.2.27 twice weekly on an escalating scale, at doses of 1, 10, 50, 100, and 200 mg, i.e., a total of only 361 mg. After the Mab was administered intravenously to eight patients with metastatic malignant melanoma, biopsies of their metastatic nodules clearly demonstrated that the Mab specifically localized in the tumors. There was also a clear dose–response relationship, since 200 mg of Mab was required to stain most of the skin nodules; however, the viable tumor cells examined by flow cytometry were clearly not saturated by Mab, suggesting that the highest dose injected was still suboptimal. Interestingly enough, Mab 9.2.27 was not toxic to any of the patients. This contrasts with results from trials to evaluate Mab T101 for treatment of patients with cutaneous T cell lymphoma and chronic lymphocytic leukemia. In that case, patients treated with 50 mg of T101 during a 2-hour infusion period developed chest tightness and shortness of breath. Although these symptoms disappeared when the rate of infusion was only 1–2 mg/hour, several patients still developed urticaria afterward. Mab T101 apparently caused pulmonary toxicity as it bound to circulating cells, leading to microaggregates in the pulmonary microvasculature (Foon *et al.*, 1983). Mab 9.2.27 does not bind to leukocytes, so it is not subject to this sequela. Only one patient developed serum sickness, which responded to steroid treatment, and circulating 9.2.27 antiglobulin complexes were not evident. In fact, only 50% of these patients developed any antiglobulin response. Even this was transient and regulated by substituting higher antibody doses. There was an apparent feedback inhibition of the antiglobulin response, since no antiglobulin appeared after the 200-mg dose. Also, pharmacokinetic studies indicated that the clearance rate of murine immunoglobulin was not affected by the transient antiglobulin responses. The data indicate that carefully prepared murine Mab can be given to immunologically responsive patients with solid tumors, without eliciting a therapeutically limiting antiglobulin response. Although there was no demonstrable tumor regression, Mab 9.2.27 clearly localized to the tumor and caused no toxic response. Most importantly, flow cytometric and other studies indicated that the treatment did not cause anti-

genic modulation on the surfaces of melanoma cells (Oldham *et al.*, 1984).

Abrams *et al.* (1985) reported on a second phase of the foregoing clinical trials with Mab 9.2.27, particularly on its localization and biodistribution in patients with melanoma. This time, saturation of all antigen-binding sites was achieved in some patients with escalation of the dose to 500 mg, i.e., patients received 1, 10, 50, 100, 200, and 500 mg of Mab 9.2.27. In a third study phase, patients received either 500 mg of antibody as a single dose, or five equally divided doses given daily with a 4-week interval to allow the clearance of all residual antibody from the tumor nodules. Both schedules produced saturation in some patients, but the first dose, regardless of the schedule, was generally better than the second in three patients whose anti-murine antibodies developed during the rest interval. In the fourth study phase, patients received 1 mg Mab 9.2.27 that was chelated to 5 mCi indium-111 with diethylenetriamine-pentaacetic acid. This radiolabeled antibody was administered to patients either alone or with the addition of 50 mg of unconjugated 9.2.27. Serum half-life of the labeled and unlabeled antibody was approximately 30 hours. The latter dose proved superior for imaging tumors, detecting masses as small as 5 × 5 mm, and occult metastases. *In vitro* studies at 37°C revealed gradual loss of indium-111 from the chelate. These data indicate that Mab 9.2.27 was safe for the patients, localized selectively in melanoma tumor nodules, and bound all available sites in these nodules at doses of 200–500 mg. However, the indium-111 label may not be optimal for this antibody, because its uptake by the reticuloendothelial system was substantial. Indeed, other radionuclides, e.g., ^{99m}Tc may prove to be a better choice for this purpose (Abrams *et al.*, 1985).

A recent report by Houghton *et al.* (1985) is most interesting, since these investigators achieved significant tumor regression in 3 of 12 melanoma patients treated in a phase I clinical trial with R24, another GD3 Mab of IgG_3 isotype. Specifically, Mab R24 was injected at doses of 8 mg/m^2 over a period of 2 weeks. Inflammatory reactions consisting of urticaria, pruritus, erythema, and subcutaneous ecchymoses were observed around tumor sites in patients treated with a total of 80 mg/m^2. As was observed in earlier clinical trials with murine Mabs (Sears *et al.*, 1982c; Foon *et al.*, 1983; Oldham *et al.*, 1984), human anti-mouse antibody appeared in the patients' sera. Peak antibody levels in sera were dose related and ranged from 0.1 to 62 μg/ml. These antibodies were detected in all patients who could be evaluated between 15 and 40 days after the start of therapy. Interestingly, much like the clinical trials with another murine anti-melanoma Mab 9.2.27

(Oldham *et al.*, 1984), Mab R24 did not provoke antigenic modulation, since cell surface expression of GD3 remained intact in the face of substantial levels of circulating and tumor-bound Mab R24.

Based on the data obtained by Houghton and colleagues (1985), one can express cautious optimism regarding the potential usefulness of such treatment. In fact, these investigators were most cautious in interpreting their own data, clearly indicating that some distant tumor metastases were not affected by the antibody, although they later revealed considerable GD3 expression. The involvement of immunological effector mechanism(s) is certainly suggested by the 4- to 12-week time intervals required before an approximately 50% decrease in the size of some lesions. The mechanisms involved may range from antibody-directed complement-mediated cytotoxicity and cell-mediated cytotoxicity to tumor cell injury secondary to the inflammatory reaction elicited in the tumor bed by Mab R24, the authors note. This observation, plus the infiltration of T cells and mast cells, mast cell degranulation, and the deposition of complement components C3, C5, and C9 in the tumor bed certainly support the contention that inflammation occurred at this site.

Another observation of Houghton *et al.* (1985) is intriguing, namely that responses to R24 treatment of melanoma tumor-bearing nude rats and mice were actually enhanced by intermittent chemotherapy with adriamycin. Such treatment may increase blood flow and alter tumor cell susceptibility to drugs as a consequence of antibody treatment. Obviously, one may also conclude that antibody–drug conjugates, especially if they involve anti-GD3 antibodies, may turn out to be a useful modality for immunotherapy of malignant melanoma.

V. Perspectives

A large body of experimental data clearly indicates that Mabs directed to antigens expressed on the surfaces of human tumor cells are excellent probes for elucidating the structure and function of these antigens and possibly for developing cancer therapies. This brief review article has attempted to document this point of view by focusing on those relatively few target antigens whose structure and function have been delineated by well-characterized Mabs. It is quite evident that at this time Mab-mediated tumor therapy is still very much in its infancy, and experimental data are simply not sufficient to warrant thorough evaluation of this potentially exciting area of research. It will be essential to obtain as much information as possible about antigens targeted by Mabs to extend our basic knowledge about tumor invasion

and metastasis. The requirement for gaining this type of basic knowledge can best be appreciated in the context that Mabs are certainly not "magic bullets," which by themselves will have a major impact on cancer therapy. Two major strategies seem to offer the best hope for making therapeutic use of these unique immunological reagents.

The first is based on the prediction made by Paul Ehrlich some 80 years ago that antibodies will serve as carriers to target drugs and toxins to tumor cells and thereby increase the chance of their ultimate eradication. There is presently a concerted effort worldwide to evaluate the efficacy of tumor cell destruction by Mab-mediated targeting of chemotherapeutic drugs, radionuclides, as well as plant toxins. Some results from preclinical trials in animal model systems warrant cautious optimism, but are too premature to yield meaningful conclusions at this time.

The second basic strategy is to strengthen the tumor host's immunological defense system by "arming" suitable effector cells for tumor cell destruction. One such effort described here is arming effector cells with specific Mabs that then effectively destroy established human melanoma and colorectal carcinoma tumors in athymic mice. Actually, a few such approaches are currently being tested clinically, and preliminary results presented very recently at the Second International Hybridoma Congress are hopeful. Another means to stimulate effector cells that kill tumors was that pioneered by Rosenberg *et al.* (1985). The cancer patients' peripheral blood lymphocytes were treated with such highly potent effector molecules as recombinant interleukin (rIL-2) to produce lymphokine-activated killer (LAK) cells that, in some instances, caused pronounced tumor regression in some patients with advanced cancer. These investigators noted that considerable refinement is needed to overcome the rather serious clinical problems evoked by this treatment. One such attempt is made in the authors' laboratory in preclinical trials, by "arming" LAK cells with suitable Mab and thereby target them to tumors.

Another interesting and novel approach designed to kill tumor cells is the use of bispecific Mabs, based on the hybrid hybridoma technology developed by Milstein and Cuello (1983). Cytotoxic T cells generated from peripheral blood mononuclear cells with OKT-3 antibody effectively lysed melanoma cell targets that were coated with bifunctional heteroconjugates consisting of OKT3 and Mab 9.2.27 (Jung *et al.*, 1986). Other hybrid hybridomas, e.g., those between Mab 9.2.27 and Mabs to murine and human T cell receptors, are also being tested for their effectiveness of tumor destruction in nude mice bearing human melanomas that metastasize spontaneously.

Clearly, these new potential cancer therapies involving Mabs to defined tumor target antigens will have to be optimized, first in animal model systems and then in phase I clinical trials. The most difficult and critically important task will then be to combine several such optimal treatment modalities in a synergistic fashion to achieve maximal tumor destruction. Considering the immense complexity and heterogeneous quality of solid tumor systems, combined therapy seems to offer the best hope for applying the research described here.

All such future achievements depend almost completely on active progress in basic research in biology, biochemistry, genetics, and immunology. Thus, it is indeed encouraging that murine Mabs, ever since their development by Kohler and Milstein (1975), combined with gene cloning technology have had a decisive impact on the most rapidly accelerating advances in biomedicine to date. There is little doubt that the new experimental approaches made possible by these powerful technologies will lead to new concepts and to a marked and sustained progress in basic cancer research.

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ADVANCES IN IMMUNOLOGY, VOL. 40

Human Marrow Transplantation: An Immunological Perspective

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I. Introduction

Studies of radiation-induced marrow chimeras have provided a vast amount of information concerning a variety of fundamental immunobiologic questions. During the past four decades, this model has served as an essential and extremely versatile tool for inquiry regarding development and differentiation of hematopoietic precursors, cellular interactions of the immune response, function of molecules encoded by the major histocompatibility complex (MHC), mechanisms of allograft rejection and tolerance, and mechanisms of autoimmunity. The understanding generated in these studies has also provided a rational foundation for development of marrow transplantation as a therapeutic modality in humans.

The first successful marrow transplants were carried out with identical twins as donor for patients with acute leukemia (1). Later development of the one-way mixed leukocyte culture (MLC) test (2) and alloantisera for HLA typing (3,4) made it possible to select MHC-identical allogeneic donor/recipient pairs. At the same time, tolerable preparative regimens of total body irradiation (TBI) and chemotherapy capable of providing sufficient immunosuppression for engraftment of allogeneic marrow were developed, and posttransplant immunosuppressive regimens allowing at least partial control of graft-versus-host disease (GVHD) were identified. Finally, supportive care with intravenous hyperalimentation (5), platelet (6) and granulocyte (7,8) transfusions, protective isolation (9), and antibiotics helped to improve survival during the early posttransplant period. Collectively, these developments have made it possible for marrow transplantation to evolve from a last-resort experimental therapy reserved for patients with end-stage leukemia or aplastic anemia to become the first-line treatment of choice for an increasingly large number of conditions.

Currently, most marrow transplants are carried out between sibling donor/recipient pairs who are genotypically identical for the HLA class I (A locus and B locus) and class II (DR locus, DQ locus) antigens of both parental haplotypes (10,11). For any given sibling pair, there is a 25% probability of HLA genotypic identity, and for any patient the probability of having an HLA-identical sibling is 40% or less (10,12). Marrow transplantation has also been carried out using HLA phenotypically identical or partially identical related or unrelated donors (13–16).

The indications for marrow transplantation depend on the underlying disease and the degree of genetic disparity between the donor and recipient (17). If an HLA-identical sibling is available, marrow transplantation is the therapy of choice for patients with severe combined immunodeficiency (18,19), severe aplastic anemia (20), acute leukemia at or beyond first relapse (21), and for chronic myelogenous leukemia (CML) (22,23). Some proportion of patients with acute nonlymphocytic leukemia (ANL) in first remission may be cured with chemotherapy alone, and the exact role of marrow transplantation in this situation remains to be defined (24,25). Approximately 50–60% of patients transplanted in the first remission of ANL have apparent cure of the disease (26,27). Marrow transplantation has also been used successfully to treat life-threatening genetic or acquired nonmalignant disorders of the marrow such as thalassemia, Fanconi's anemia, osteopetrosis, congenital aregenerative anemias and acute myelofibrosis, and life-threatening metabolic abnormalities such as Gaucher's disease and mucopolysaccharidoses (28,29).

The indications for marrow transplantation are more limited if an HLA-identical sibling is not available. Because of the increased risk of complications such as rejection or GVHD (14), marrow transplantation from a partially compatible donor should not be recommended for patients with CML in chronic phase or ANL in first remission where there is a good prognosis for the immediate future or where the disease may be cured by conventional therapy. Transplantation from a partially compatible donor is justified for patients with more advanced phases of these diseases because of their rapidly progressive character and lack of curability with conventional therapy. The little experience to date with partially compatible transplants for patients with aplastic anemia and other nonmalignant diseases has generally been unfavorable, although new conditioning regimens and T cell depletion of donor marrow are being explored as possible methods for improving results.

Patients with severe combined immunodeficiency given allogeneic marrow and aplastic patients given identical twin marrow can sometimes be engrafted with no pretransplant preparative regimen, although certain exceptions have been reported (30,31). For patients with leukemia, engraftment of HLA-identical marrow is reliably achieved with preparative regimens of cyclophosphamide (60 mg/kg on each of two successive days) and TBI, generally administered in a fractionated schedule to decrease toxicity to the gastrointestinal tract, liver and lungs (32,33). Because leukemic relapse represents the main cause of failure, a large number of alternative preparative regimens have been tested (21,27,34–40). Most studies have a one-arm design, making it difficult to evaluate results. Until recently, most attempts to decrease relapse rates by increasing the vigor of the pretransplant regimen have been accompanied by increased toxicity and little, if any, survival benefit. For most patients with aplastic anemia, durable engraftment of HLA-identical marrow can be achieved with a preparative regimen of cyclophosphamide (50 mg/kg on each of four successive days) (20). There is an appreciable risk of graft rejection in certain subgroups and other preparative regimens have been tested (see below).

The outcome of transplantation for leukemia depends in large part on the state of the underlying disease. Patient with ANL at or before second remission (26,27) or CML at or before acceleration (22,23) have a 3-year relapse probability of 25–40%. Patients with more advanced stages of these diseases have a 3-year relapse probability of 60–80% (41). For all patients, there is an age-related risk of other fatal complications, including venoocclusive disease of the liver (42,43) or other toxicities of the preparative regimen, idiopathic or cytomegalovirus (CMV) interstitial pneumonia (44), and infections with or without GVHD. For otherwise healthy younger patients (less than 20 years of age) receiving marrow from an HLA-identical sibling for treatment of early-stage leukemia, there is a 20–30% risk of a nonleukemic fatal complication during the first 6 months after transplantation. Older patients, those with late-stage leukemia, and those with partially matched donors have proportionately higher risks of fatality not due to leukemia. Fatal complications are less frequent in patients transplanted for aplastic anemia and are generally caused by infections with or without GVHD (20).

Given the above general overview, the remainder of this article will focus in detail on the four principal immunologic aspects of human marrow transplantation: hematopoietic engraftment, GVHD, immuno-

logic reconstitution, and the graft-versus-leukemia effect. Each of these immunologic aspects has considerable impact on the course and outcome of marrow transplantation in humans. Graft rejection, when it occurs, is nearly always a fatal complication. Second transplants have been attempted but are seldom successful. GVHD itself seldom causes death but is often associated with fatal infections such as CMV interstitial pneumonia. The immunodeficiency that occurs after marrow transplantation does not completely correct itself for at least a year and underlies much of the morbidity and mortality of the procedure. Finally, as discussed above, recurrent disease is still the single most frequent cause of failure in patients transplanted for leukemia.

II. Hematopoietic Engraftment

Marrow for transplantation is obtained by multiple aspirations from the posterior iliac crests and contains on the order of $10–40 \times 10^9$ nucleated cells in a volume of 500–800 ml, composed mainly of erythrocytes and plasma (45). Ordinarily, the only processing is passage through a sieve screen to remove large particulate material before intravenous administration to the recipient. ABO incompatibility may be overcome either by plasmapheresis to decrease the isohemagglutinin titer (46–49) or by further processing of the donor marrow to remove the incompatible erythrocytes (50,51). The transplanted marrow comprises a mixture of cells at various stages of development within the erythroid, myeloid, monocytic, megakaryocytic, and lymphoid lineages (52) together with stromal cells (53). Hematopoietic cells arise from a small population of undifferentiated self-renewing stem cells (52) which likely constitute the only essential part of the graft. Progeny of these stem cells generally begin to appear in the marrow and blood of the recipient between 2 and 3 weeks after transplantation. In successful grafts, hematopoiesis appears entirely normal and persists indefinitely with only cells of donor origin detectable in most patients (21); persistence of host cells can occur in some patients, resulting in "mixed chimerism" (54,55).

The term "graft failure" encompasses several clinical syndromes having diverse etiologies. Failure of initial engraftment (primary graft failure) can be caused by unappreciated genetic disparity between the donor and recipient, inadequate immunosuppression of the recipient, unappreciated abnormalities or insufficient numbers of stem cells in the graft, abnormal marrow microenvironment in the recipient (e.g., due to myelofibrosis) (56), drug toxicity (see below), or possibly viral infection. Graft failure occurring after initial engraftment (secondary

graft failure) can also have multiple causes. For example, antibiotics such as trimethoprim-sulfa, anti-viral agents such as α-interferon, adenine arabinoside, and 9-(1,3-dihydroxy-2-propoxymethyl) guanine (DHPG), and possibly methotrexate given for prophylaxis of GVHD or antithymocyte globulin given for treatment of GVHD (57) can produce marrow damage as direct, idiosyncratic, or possibly allergic effects. Viral infections may depress hematopoietic stem cells either by direct or indirect effects. Aplastic anemia can occur following infection with hepatitis B virus, and CMV infection after marrow transplantation is sometimes myelosuppressive (58). In certain clinical situations, and in animal models where parental marrow is given to unirradiated F_1 hybrids, GVHD can cause aplasia (59,60).

The morphologic changes that accompany graft failure are nonspecific (61). Usually the problem comes to attention because of decreasing leukocyte counts which can initially involve isolated neutropenia, thrombocytopenia, or reticulocytopenia, or may simultaneously involve all three lineages. Occasionally, a marked atypical lymphocytosis or marrow plasmacytosis may herald the onset of graft failure. Examination of the marrow usually shows hypocellularity which can be nonuniform in distribution and sometimes accompanied by nonspecific changes suggestive of damage, such as fat necrosis, granulomas, congestion, hemorrhage, or erythrophagocytosis. Occasionally, silent graft rejection occurs and is detected only by examining genetic markers of donor or host origin in marrow cells (62).

Disappearance of donor cells accompanied by reappearance of host cells in the marrow is usually taken as the hallmark of immune-mediated graft rejection. Three settings have been identified in which an increased risk of graft failure has a well-defined immunologic basis. These include multiply transfused patients with aplastic anemia, patients receiving MHC-mismatched marrow, and patients receiving T cell-depleted allogeneic marrow. Each of these three settings will be discussed in turn together with relevant results from animal experiments.

A. Graft Rejection in Patients Transplanted for Aplastic Anemia

By the mid-1970s, marrow transplantation was established as the therapy of choice for aplastic anemia in patients under the age of 40 who have an HLA-identical donor (63–66). Before 1975, marrow graft rejection occurred in 30–60% of patients prepared with the standard cyclophosphamide regimen. A binary logistic regression model identified two factors that strongly correlated with graft rejection (67,68):

reactivity between patient and donor cells in MLC, and a low number of donor marrow cells ($< 3 \times 10^8$ cells/kg recipient) used for transplantation. Low-level but significant MLC reactivity between HLA-identical siblings was interpreted to result from transfusion-induced allosensitization to minor histocompatibility antigen (see below). Three approaches were undertaken in attempts to decrease rejection in aplastics: (1) transplantation before transfusion-induced allosensitization, (2) transplantation of larger numbers of cells obtained from donor buffy coat, and (3) the use of more immunosuppressive conditioning regimens.

Studies in experimental animals suggested that random blood transfusions increased the risk of graft rejection after marrow transplantation from an MHC-identical donor (see below). Subsequent clinical studies confirmed the prediction that transplantation before blood transfusion would reduce the risk of graft rejection (69,70). In the Seattle experience, only 4 of 50 untransfused aplastic patients rejected marrow from an HLA-identical donor (70). Survival for this group of patients is projected to be more than 80% at 10 years after transplantation.

Attempts to improve results in transfused patients through the use of donor buffy coat were prompted by several observations. First, circulating stem cells have been shown to exist in several species, including mice, guinea pigs, dogs and nonhuman primates (71–75). Second, peripheral blood lymphocytes enhance canine and human *in vitro* hematopoiesis (76,77). Third, thymocytes, peripheral blood leukocytes, and thoracic duct lymphocytes enhance allogeneic marrow engraftment in mice and dogs (78–80). Clinical studies have shown that the addition of buffy coat infusions after marrow transplantation reduced the risk of rejection, thereby increasing survival in transfused recipients. In the original study, only 6 of 43 patients who received donor buffy coat had rejection, compared to 7 of 22 patients who received only marrow (81). Currently 65% of the patients who received buffy coat are alive, compared to 50% in patients who received only marrow.

More intensive immunosuppressive conditioning regimens have also been explored for transfused patients. Regimens that employ cyclophosphamide and TBI appear to reduce the incidence of rejection, but survival is often not improved mainly because of increased mortality from GVHD and interstitial pneumonia (82,83). Regimens of cyclophosphamide and total lymphoid irradiation (TLI) or thoracoabdominal irradiation with lung shielding have been tested in younger patient populations and were associated with decreased rejection and

projected survivals of approximately 70% (84–86). Enthusiasm for regimens that include radiation together with chemotherapy must be tempered by concerns regarding increased cancer risk, sterility, and effects on growth and development. One study has suggested that the rejection rate is decreased in patients given cyclosporine rather than methotrexate for GVHD prophylaxis (87).

A study by Hill *et al.* (88) has analyzed the incidence and biologic impact of mixed chimerism after transplantation of HLA-identical marrow in aplastic patients prepared with cyclophosphamide. Host lymphoid or hematopoietic cells were detected by cytogenetic analysis on one or more occasions after transplantation in nearly 60% of the patients. Mixed chimerism was not influenced by either pretransplant transfusion or by posttransplant donor buffy coat infusions. Graft rejection, which occurred mostly in transfused patients, was seen in 30% of patients who had mixed chimerism and in only 5% of patients whose blood and marrow cells were entirely of donor origin. Patients with mixed chimerism who did not reject their grafts had gradual disappearance of host cells and only one patient has stable mixed chimerism persisting for more than 1 year after transplantation. The increased incidence of graft rejection among mixed chimeras may reflect host resistance to the immunosuppressive effect of cyclophosphamide, or an inability of donor cells to mount an anti-host immunological response. The latter could occur if minor histocompatibility antigens of the donor and recipient were closely matched or if donor cells lacked immune response genes for minor histocompatibility antigens of the recipient. Host cells not eliminated by the cyclophosphamide or by donor cells might subsequently cause rejection.

A similar study of chimerism has been carried out in leukemic patients prepared for transplantation with cytosine arabinoside, cyclophosphamide, and 10.0 Gy TBI or with 13.2 Gy TBI and either cyclophosphamide or VP-16 (89). There was a 24% incidence of mixed chimerism with the first regimen and a 12% incidence with the second regimen. Some patients remained mixed chimeras for up to 70 months, unlike the aplastic patients reported by Hill *et al.* (88). Patients with mixed chimerism did not have graft failure and did not have a higher leukemic relapse rate than patients who had only donor-type hematopoietic cells. The incidence of acute GVHD was similar in the two groups, but patients with mixed chimerism had significantly better survival than patients in whom host hematopoietic cells were not detected. Thus, the biologic significance of mixed chimerism in marrow transplant recipients may depend on the underlying disease.

B. Experimental Studies of Transfusion Effects

Studies in the canine model established that pretransplant transfusions of whole blood from either the intended donor or from DLA-nonidentical random donors increased the incidence of rejection in lethally irradiated (9.2–12.0 Gy) recipients given marrow from DLA-identical littermates (90–92). Marrow graft rejection occurred in all dogs given DLA-identical littermate transplants after three transfusions of whole blood from the donor. Subsequent studies in the canine model indicated that blood leukocytes, but not platelets or erythrocytes, were responsible for the transfusion effect (93). Transfusion of cotton wool nonadherent peripheral blood cells, granulocytes, or thoracic duct lymphocytes did not cause increased rejection (94). The cotton wool nonadherent blood cells were virtually free of monocytes and largely depleted of Ia-positive cells, suggesting that Ia-positive cells might be required to induce a response in the recipient. Taken together, the findings suggest the hypothesis that transfused Ia-positive cells induce a response to certain non-DLA minor histocompatibility antigens, causing the generation of immune cells that can survive total body irradiation. In support of this hypothesis are findings that graft rejection was not increased by transfusions of UV-irradiated whole blood containing mononuclear cells unable to stimulate MLC responses or provide accessory function for mitogen-stimulated cultures (95).

C. Engraftment of HLA-Incompatible Marrow

In the Seattle experience, HLA incompatibility has been a major factor associated with primary or secondary graft failure in patients with aplastic anemia. Six of 11 aplastic patients prepared with various nonradiation immunosuppressive regimens did not engraft after transplantation of marrow from a haploidentical family member incompatible for one or two HLA antigens (96). Two other patients had initial engraftment with subsequent rejection. Five of the six patients with failure of engraftment and one of the patients with graft rejection were untransfused before transplantation.

A significant, though less striking, increase in primary and secondary graft failure has been reported for leukemic patients prepared with cyclophosphamide and TBI and transplanted with HLA-incompatible marrow (14). Five of 105 patients receiving marrow from a donor other than an HLA-identical sibling had frank graft rejection, whereas only 1 of 728 leukemic patients receiving marrow from an HLA genotypically identical sibling had graft rejection documented by return of

host cells. The risk of rejection increases proportionately with the degree of HLA incompatibility. Problems with engraftment of HLA-incompatible marrow are also evident when time to recovery of hematopoiesis is analyzed. Fifteen percent of patients grafted from a one-locus incompatible donor fail to reach an absolute granulocyte count of 1000/mm^3 by day 40 after transplantation (unpublished).

D. Marrow Graft Resistance

The term "marrow graft resistance" has been used to describe failure of engraftment or marrow graft rejection unrelated to previous alloimmunization. In murine models, graft resistance is usually assessed by measuring hematopoietic activity in the spleen early after transplantation and shows two patterns. Failure of engraftment can occur in irradiated recipients in certain allogeneic combinations ("allogeneic resistance") (97) or in irradiated F_1 hybrid recipients given parental marrow ("hybrid resistance") (98). Both types of resistance are strain dependent (99) and show linkage to H-2D genes of the MHC (100,101). To a limited extent, allogeneic resistance has also been linked to H-2K and I genes (102). Hybrid resistance is thought to involve recognition of "hybrid histocompatibility" (Hh) antigens. The mechanism of marrow graft resistance is distinct from conventional allograft responses. Resistance is mediated by bone marrow-derived (103,104), thymus-independent (97,98,105), radioresistant (97,98,106) cells. Destruction of grafted hematopoietic stem cells occurs rapidly without prior sensitization or priming by donor antigens. In the mouse, graft resistance can be ablated by pretreatment of the recipient with fractionated radiation (97,98,107), cyclophosphamide (97,98), silica particles (99), carrageenan (99,108), or glucan (109) and by infusion of donor thymocytes (110).

Several lines of evidence have directly implicated natural killer (NK) cells in marrow graft resistance. Treatment of recipients with an antiserum specific for NK cells (111) ablated marrow allograft resistance, and injection of recipients with NK clones caused allograft rejection in NK-deficient beige mice which do not manifest marrow graft resistance (112). The nonspecific character of NK cytotoxicity was difficult to reconcile with observations that cells mediating allogeneic resistance show a high degree of specificity. It has been shown that antibody directed against H-2D gene products can account for this specificity due to the participation of NK cells in antibody-dependent cell-mediated cytotoxicity (113,114). On the other hand, F_1 recipients can reject parental marrow in the apparent absence of any detectable antibody, suggesting the possible involvement of other mechanisms of recognition.

Hematopoietic grafts can also be destroyed by alloimmune responses (115). NK-deficient beige mice could be primed for specific marrow graft rejection by injection of allogeneic tumor cells, and pretransplant injection with cytotoxic T cell clones caused specific rejection in irradiated recipients. Similar strategies for priming or adoptive transfer could not induce rejection of parental marrow by F_1 hybrid recipients. An unexpected result was that mice irradiated (7.0 Gy) and given allogeneic marrow showed specific rejection of second-set allografts after reirradiation. This finding suggested that some T cells survived the radiation and retained the capacity to develop a functional response. It was suggested that graft rejection mediated by NK cells and occurring within 24 hours after transplantation might not allow for sensitization and participation of T killer cells, but that rejection at 3 days or later may well involve T cell sensitization and T cell-mediated cytotoxicity.

Canine models of marrow graft resistance have been developed with both DLA-identical and -nonidentical transplantation (116). Recipients given single-dose TBI (9.2 Gy) and DLA-identical littermate marrow grafts containing $0.5–4 \times 10^8$ cells/kg recipient weight almost uniformly achieve engraftment. Recipients given dimethylbusulfan at 10 mg/kg instead of TBI often had graft rejection occurring between 11 and 63 days after transplantation (117). Rejections were frequently preceded by a host-derived lymphocytosis. In this model, rejection could be prevented by conditioning recipients with anti-thymocyte globulin (ATG) in addition to dimethylbusulfan.

Recipients given single-dose TBI (9.2 Gy) and marrow in low cell doses ($0.5–4 \times 10^8$ cells/kg) from a DLA-nonidentical littermate or unrelated donor almost uniformly have failure of engraftment, whereas 50% of recipients given marrow from DLA phenotypically identical donors achieve engraftment (118). These results suggest that some of the antigens recognized by cells capable of causing rejection are encoded or controlled by genes within or near the histocompatibility complex. Cells responsible for rejection of DLA-nonidentical marrow are highly radioresistant (119). Reliable engraftment was possible only when the TBI dose was increased to 18 Gy. Treatment of canine recipients with *Corynebacterium parvum* or anti-macrophage agents such as silica particles did not uniformly prevent rejection (120,121). Unlike cells sensitized by transfusion and cells that mediate rejection of DLA-identical marrow grafts, the cells that mediate rejection of DLA-nonidentical grafts were not sensitive to anti-thymocyte serum (with or without procarbazine). Pretransplant or early post-transplant administration of a murine monoclonal antibody (0.2–0.4

mg/kg/day for 5 days) that reacts with an epitope of class II molecules not recognized by antibodies in the anti-thymocyte globulin did result in sustained engraftment in almost half of the recipients (122). Finally, administration of methotrexate after transplantation enhanced engraftment of DLA-nonidentical marrow, suggesting that cells involved in graft rejection undergo rapid proliferation after TBI and allogeneic marrow transplantation (123).

E. Graft Rejection in Patients Receiving T Cell-Depleted Marrow

Depletion of T cells from donor marrow offers a distinct alternative method for preventing acute GVHD. Transplantation of T cell-depleted marrow, however, has led to a new problem. Acute rejection occurring after an initial period of engraftment has become a major complication. In one study, 11 patients with chronic myelogenous leukemia in chronic phase or acute nonlymphocytic leukemia in first remission received 12.0 Gy of fractionated TBI and HLA-identical marrow containing a total of approximately 1×10^6 T cells (124). Six patients developed irreversible graft failure between days 21 and 244 after transplantation, one developed graft failure that reversed spontaneously, and one showed evidence of hematopoietic and lymphoid mixed chimerism in cytogenetic studies. Among 41 patients with advanced hematologic malignancies who received 15.75 Gy of fractionated TBI and T cell-depleted marrow, 7 had graft failure and nearly all patients evaluated showed evidence of mixed hematopoietic and lymphoid chimerism. The cumulative incidence of graft failure with the different TBI regimens was 69 and 25%, respectively (Fig. 1). No relationship has been seen between failure of T cell-depleted grafts and the number of nucleated cells or hematopoietic precursors assayed as BFU-E or CFU-C in the graft (125). Similarly, prior transfusions and donor or recipient sex had no effect on graft failure. Postgrafting immunosuppression with regimens of cyclosporine alone, methotrexate alone administered on days 1, 3, 6, and 11, or cyclosporine combined with methotrexate administered on days 1, 3, 6, and 11 did not prevent graft failure in leukemic patients receiving HLA-identical T cell-depleted allogeneic marrow after preparation with cyclophosphamide and 15.75 Gy of fractionated TBI (unpublished).

Evidence suggests that damage to stem cells during T cell depletion was not the major cause of graft failure. (1) In most patients, graft failure occurred after initial engraftment was well established. Stem cell damage would ordinarily be expected to manifest itself as failure of initial engraftment. On the other hand, marrow treatment could

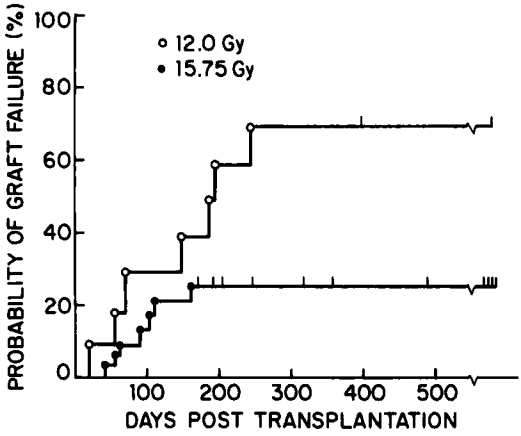

FIG. 1. Probability of graft failure in patients receiving T cell-depleted HLA-identical allogeneic marrow transplants after preparation with cyclophosphamide (120 mg/kg) and either 12.0 (○) or 15.75 (●) Gy of fractionated TBI (p = 0.032, log-rank test).

conceivably have limited the self-renewal of stem cells or caused a differential toxic effect on the least mature stem cells required for durable engraftment without affecting more mature stem cells required for initial engraftment. (2) Graft failure has not occurred in any of seven evaluable patients who received autologous grafts depleted of T cells by the same method (unpublished). On the other hand, this might merely reflect the lower dose of stem cells required for autologous engraftment. (3) The significant inverse association of graft failure with TBI dose would not be expected if damage to stem cells were the only cause of graft failure and suggests a major role for host factors.

In a formal sense, the above results could not distinguish whether host factors were affected by the radiation per se or by the underlying diagnosis and prior treatment. Data from other centers, however, have confirmed the influence of the pretransplant radiation regimen on the risk of graft failure after transplantation of T cell-depleted marrow. Patterson *et al.* (126) observed only 1 rejection among 37 patients receiving HLA-identical T cell-depleted allogeneic marrow after preparation with 7.5 Gy single-dose TBI delivered at 26 R/min. In contrast, 8 of 13 patients receiving identically treated marrow after preparation with fractionated TBI (10.0–12.0 Gy) had primary or secondary graft failure.

Numerous studies have now reported an increased incidence of graft failure in patients receiving marrow depleted of T cells by a variety of methods (124–133). Data suggest that the risk of graft failure

depends on multiple variables, including the primary disease, the degree of genetic disparity between the donor and recipient, the potency of the pretransplant immunosuppressive regimen, and the number of residual T cells in the graft. Engraftment with T cell-depleted marrow may be more readily achieved in children with severe combined immunodeficiency than in patients with leukemia or aplastic anemia (133). Increased genetic disparity at MHC loci has been associated with an increased risk of graft failure (131). In one study, the addition of cytosine arabinoside to a regimen of cyclophosphamide plus TBI decreased the incidence of graft failure in patients receiving T cell-depleted MHC-mismatched marrow (134). Finally, Herve *et al.* (135) found no graft failures among 33 evaluable leukemic patients who received marrow that contained approximately 50×10^6 total T cells. Determination of the relative importance of the above variables will require strictly controlled protocols in patients where chimerism of both myeloid and lymphoid cells is carefully documented.

The data suggest that certain T cells in the donor marrow serve a beneficial function by helping to sustain engraftment. It is conceivable that T cells or T cell-generated factors are necessary for sustained proliferation of hematopoietic precursors and that T cells regenerated in the host environment are inadequate in number or function to mediate this effect. This hypothesis does not readily explain the observed inverse relationship between radiation dose and graft failure or the absence of graft failure in patients receiving T cell-depleted autologous grafts. Alternatively, donor T cells may be required to eliminate residual host cells in order to provide an optimal microenvironment for growth of donor-derived precursors. This hypothesis, however, implies that genetic identity between stem cells and relevant accessory cells is necessary for sustained hematopoiesis. The data considered as a whole best fit the hypothesis that certain T cells in the donor marrow facilitate durable engraftment by eliminating host cells that can cause graft failure.

F. Facilitation of Engraftment by T Cells

Studies by Goodman *et al.* indicated that donor thymocytes but not lymph node lymphocytes enhanced engraftment of parental marrow in irradiated F_1 hybrid recipient mice (78,136,137). Compared to parental thymocytes, F_1 thymocytes had only a relatively modest effect. Thymocytes from $P \rightarrow F_1$ irradiation chimeras were more effective than parental thymocytes in augmenting hematopoiesis, despite a complete inability to cause acute GVHD (78). Thymocytes semisyngeneic to both the parental marrow donor and the F_1 hybrid recipient did not

facilitate engraftment despite the ability to produce acute GVHD. Thus there was strong evidence that the marrow graft enhancing effect of parental thymocytes was completely unrelated to GVHD. In fact, in the same experiments it appeared that GVHD had an inhibitory effect on hematopoiesis.

A graft-facilitating role for T cells has also been shown by Soderling *et al.* (138), who found durable engraftment of donor-type blood mononuclear cells in most C57BL/6 mice prepared with 13.2 Gy fractionated TBI (1.65 Gy per fraction), 7.5 Gy single-dose TBI, or with 300 mg/kg cyclophosphamide plus 9.0 Gy TLI, and given 25×10^6 BALB/c marrow cells. Graft failure was frequent in mice prepared similarly and given Thy-1-depleted marrow and spleen cells. Durable engraftment with Thy-1-depleted cells could be achieved in mice prepared with 9.0 Gy single-dose TBI, 13.2 Gy fractionated TBI (3.3 Gy per fraction), or with two doses of 120 mg/kg cyclophosphamide plus 7.5 Gy TBI. Ferrara *et al.* (139) found that the dose of T-depleted marrow required to achieve reliable hemopoietic engraftment (assayed by a hemoglobin marker) was directly related to the degree of genetic disparity. For a given degree of genetic disparity, reliable hemopoietic engraftment after lower doses of T-depleted marrow required higher doses of TBI. A striking and important finding was that nearly all mice had varying degrees of mixed lymphoid chimerism, whether hematopoietic engraftment had occurred or not.

In the canine model, engraftment of DLA-nonidentical marrow from unrelated donors can be facilitated by infusions of donor leukocytes (79,120). This beneficial effect did not occur when donor cells were irradiated, indicating that antigen overload alone was not the mechanism. Graft enhancement was also observed following infusions of donor thoracic duct lymphocytes (80) which, unlike peripheral blood leukocytes, do not contain hematopoietic stem cells (140). Finally, the effect of donor leukocyte infusions was diminished when recipients were given cyclosporine (123). Thus, findings in human marrow transplantation that T cell depletion increases the risk of graft failure are consistent with findings in animal models that T cells can facilitate marrow engraftment. Findings that more vigorous pretransplant conditioning can facilitate engraftment of T cell-depleted marrow are also similar in humans and in animal models.

G. A Reconsideration of Marrow Graft Failure in Humans

The relative importance of T cells and stem cells for overcoming graft resistance has not been fully defined since the experiments with T cell-depleted grafts may have been confounded by the possible

effects of small numbers of residual T cells. Similarly, the relationship between marrow cell dose and transplant outcome in transfused aplastic patients might reflect the number of T cells rather than the number of hematopoietic stem cells in the graft. Indeed, the typical pattern of rejection following prompt engraftment suggests that the number of stem cells was sufficient. Likewise, the effect of donor buffy coat transfusions may be almost entirely due to the anti-host effect of T cells. Dose–response and mixing experiments with highly purified populations of hematopoietic stem cells and T cells will be necessary in order to resolve this question in animal models.

The nature of the cells that mediate graft rejection emerges as a major question for human marrow transplantation, especially in view of attempts to prevent GVHD by T cell depletion of donor marrow. Unidirectional anti-donor reactivity in MLC and cytotoxic T cell generation has been detected in some aplastic patients who have rejected HLA-identical marrow (62). Antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity against donor cells has also been reported in aplastic patients after graft rejection (141). Kernan *et al.* (142) were able to recover phenotypically mature T lymphocytes of host origin having donor-specific cytolytic activity from patients who rejected T cell-depleted HLA-nonidentical marrow grafts. Residual host cells cultured from patients who rejected T cell-depleted HLA-identical marrow grafts had broadly reactive cytotoxicity similar to natural killer cells. On the other hand, T cells capable of HLA-class II-restricted inhibition of *in vitro* hematopoiesis have also been recovered from otherwise healthy recipients of HLA-identical marrow grafts (142a). The interpretation of these *in vitro* findings remains uncertain.

The mechanisms of rejection in irradiated recipients of HLA-identical T cell-depleted marrow and the relevance of various animal models remain obscure. The observations of Kernan *et al.* are provocative in view of the involvement of NK cells in murine models of marrow graft resistance. There is evidence demonstrating that certain large granular lymphocytes can inhibit *in vitro* hematopoiesis (143,144), an effect possibly mediated by contact-triggered release of cytotoxic lymphokines (145–147). On the other hand, mechanisms involved in late rejection of HLA-identical T cell-depleted marrow may be distinct from those involved in murine marrow graft resistance, which manifests itself as failure of engraftment. A model of late marrow graft regression has been developed in F_1 hybrid mice, but has not been extensively studied (148). Late regression of established marrow grafts was not associated with the homozygous H-2 phenotype

of donor cells (149) and could be overcome by 8.0–9.0 Gy of irradiation (150). Thus, short-term resistance and regression of established grafts appear to be independent phenomena in murine models.

The mechanisms by which donor T cells can facilitate engraftment and overcome resistance also remain obscure. The findings of Goodman *et al.* (78) demonstrate that in at least one model, cells that facilitate engraftment can be distinguished from cells that cause GVHD. In vivo recognition of marrow cells could have been competitively inhibited by an antigen overload effect of thymocytes, since Daley and Nakamura (151) have found that heavily irradiated donor strain tumor cells could facilitate engraftment of parental marrow in F_1 hybrid recipients. However, Goodman *et al.* (136) found that the effect of thymocytes was inhibited by irradiation.

One intriguing possibility is that "veto" cells account for the anti-host effect that facilitates durable engraftment. Veto cells represent effector cells that can be activated when recognized by other T cells. Following specific recognition of target antigen on the veto cell, the attacking cell is itself suppressed or eliminated (152–159). The passively recognized veto cells need not have receptors specific for antigens expressed by the cells they suppress. Veto cells of donor origin might help to sustain engraftment by "vetoing" the activity of host cells capable of causing graft rejection. The data of Goodman *et al.* could be explained if veto cells among the parental thymocytes were able to eliminate or suppress F_1 recipient NK cytotoxic cells that recognize Hh antigens. The plausibility of this concept would be enhanced by studies demonstrating that NK cells are susceptible to veto activity. As discussed below, veto cells of host origin might help to suppress GVHD.

Studies of irradiated mice reconstituted with mixtures of T cell-depleted marrow from syngeneic and MHC-incompatible donors may help to clarify the respective roles for host cells that mediate resistance and donor cells that overcome resistance (160,161). In this model, recipients become mixed chimeras tolerant to the donor as assessed by skin grafting and by *in vitro* assays. If T cells are not depleted from the syngeneic component of the graft, mixed chimerism and tolerance do not develop. Depletion of syngeneic Lyt-2^+ cells, but not L3T4^+ cells, was sufficient to allow partial engraftment of donor cells. These results suggest that Lyt-2^+ cells are capable of mediating or triggering alloresistance to marrow engraftment. Other evidence has suggested, however, that cells of both the Lyt-2^+ and L3T4^+ subsets are independently capable of causing graft rejection (161a). Engraftment of T cell-depleted marrow could not be achieved

in sublethally irradiated (6.0 Gy) mice pretreated with either an L3T4 antibody or an Lyt-2 antibody, but was readily achieved in recipients pretreated with both antibodies. These results may suggest useful approaches to the problem of graft failure in human marrow transplant recipients.

III. Graft-versus-Host Disease

A. Pathogenesis of Graft-versus-Host Disease

A syndrome originally termed "secondary disease" and now recognized as GVHD and associated infections was first described by Barnes and Loutit (162) as a cause of mortality in irradiated mice after transplantation of allogeneic hematopoietic cells. Long-term survival could be achieved only in recipients of syngeneic grafts. Further studies by Trentin (163) showed that secondary disease occurred in F_1 hybrid recipients given parental marrow but not in parental recipients given F_1 hybrid marrow. These observations led to the conclusion that secondary disease was the consequence of a donor-mediated anti-host reaction and not the result of a host-mediated anti-donor reaction. Acute GVHD represents an alloimmune reaction that does not occur when the graft is devoid of immunocompetent T lymphocytes. Thus allogeneic fetal liver cells (164) or spleen cells from neonatally thymectomized mice (165) can reconstitute hematopoiesis in lethally irradiated recipients without causing GVHD. Furthermore, GVHD does not occur when mature T cells are eliminated from the graft with the use of anti-Thy 1 heteroantisera or monoclonal antibodies (166–169). Although donor T cells are required for the initiation of GVHD, the status of the host is also a critical factor. GVHD does not occur unless the immune responses of the recipient are absent or effectively suppressed for some period of time. The former situation occurs in children with severe combined immunodeficiency and in F_1 hybrid mice given parental marrow.

The requirements for a classical graft-versus-host reaction were formulated by Billingham (170) as (1) genetically determined histocompatibility differences between the donor and recipient, (2) the presence of immunocompetent cells in the graft which can recognize foreign histocompatibility antigens of the host and mount an immunologic reaction, and (3) inability of the host to react against or reject the graft. Syndromes pathologically similar to GVHD can occur in settings that do not fulfill the above requirements. Skin reactions have been reported in humans given cryopreserved autologous marrow or syngeneic twin marrow (171–175).

A model of syngeneic GVHD has been described in rodents (176). Lethally irradiated rats given cyclosporine after transplantation of syngeneic marrow developed GVHD after cyclosporine was discontinued. Furthermore, cells mediating GVHD in this model could be adoptively transferred to irradiated syngeneic recipients. Cheney and Sprent (177) have found that administration of cyclosporine caused severe thymic medullary atrophy in mice. In particular, there was a marked decrease in the density of Ia-positive cells in the thymic medulla. These findings suggested the hypothesis that cyclosporine interferes with the antigen-presenting and tolerance-inducing function of Ia-positive cells, allowing the production of nontolerant T cells which cause GVHD when cyclosporine is discontinued. Although cyclosporine-associated GVHD does not occur in other species, the findings prove that histocompatibility differences are not absolutely necessary for the development of GVHD in rodents. Furthermore, these observations imply that GVHD in allogeneic marrow transplant recipients might result not only from alloreactive T cells in the donor marrow but also from the functional equivalent of self-reactive cells generated in the recipient after transplantation.

Two distinct forms of GVHD have been recognized both in humans and in animal models. Acute and chronic GVHD have different onset as well as different clinical and pathological manifestations. Inflammatory destruction of epithelial cells in the skin, liver, and gastrointestinal tract by cytotoxic lymphocytes represents the characteristic feature of acute GVHD, whereas chronic GVHD is characterized primarily by increased collagen deposition resulting in fibrosis. Evidence from a murine model that closely mimicks human disease has suggested that acute and chronic GVHD represent two separate pathophysiologic entities rather than different phases of a single pathophysiologic process (177a,b). T cell clones recovered from spleens of mice with chronic GVHD due to non-MHC disparity were noncytotoxic L3T4$^+$ cells which showed proliferative responses specific for autologous class II antigens and produced factors that stimulated collagen synthesis. T cell clones recovered from spleens of mice with acute GVHD due to non-MHC disparity were heterogeneous. A large proportion were cytotoxic Lyt-2$^+$ cells which showed MHC-restricted proliferative responses specific for recipient minor histocompatibility antigens and variably produced factors that stimulated fibroblast proliferation and collagen synthesis. Some of the clones had characteristics identical to those from mice with chronic GVHD, suggesting that events leading to chronic GVHD may begin soon after transplantation.

B. Acute Graft-versus-Host Disease

1. Clinical Manifestations

Acute GVHD in humans typically develops within the first 60 days after marrow transplantation and usually manifests itself as a characteristic dermatitis accompanied in its more severe forms by hepatitis and enteritis (178). Skin involvement most often initially appears as a pruritic, macular exanthem of the palms and soles with morbilliform lesions on the extremities, trunk, and face. In progressive disease, the lesions become confluent leading to generalized erythroderma, bulla formation, bacterial superinfection, and exfoliation.

Liver involvement is indicated by increased serum bilirubin and alkaline phosphatase with less striking transaminase elevation. Hepatic failure with encephalopathy, ascites formation, and prolonged prothrombin time can occasionally occur in patients with very severe hepatic GVHD. It is difficult if not impossible to assess hepatic GVHD solely on clinical grounds in patients who have concurrent viral hepatitis or a syndrome called venoocclusive disease, a toxic reaction caused by high-dose chemotherapy and irradiation. Liver biopsy can be helpful, but often cannot be performed because of clinical contraindications. Serum bilirubin provides a useful measure of severity in patients without other hepatic disease but can be misleading in patients with renal impairment.

Gastrointestinal involvement is indicated by watery diarrhea and malabsorption accompanied in more severe disease by crampy abdominal pain, anorexia, nausea and vomiting, gastrointestinal hemorrhage, and ileus. Barium contrast studies show a rapid transit time together with signs of thickening and edema in the small bowel (especially the distal ileum) and colon. In certain cases it can be difficult to distinguish GVHD from viral enteritis, and biopsy is often helpful. The volume of diarrhea provides a useful measure of severity in patients without other underlying gastrointestinal disease. Accurate assessment of hepatic or enteric GVHD in patients without skin involvement usually requires biopsy evaluation.

2. Pathologic Manifestations and Pathogenesis

The pathologic diagnosis of cutaneous GVHD is difficult until 2 to 3 weeks after transplantation, when changes induced by chemotherapy and irradiation are resolving. Grade I disease of the skin is characterized by relatively nonspecific epidermal basal cell vacuolization accompanied by a mononuclear cell infiltrate around superficial venules or in the dermis (179). These changes are also found in grade II dis-

ease together with the distinctive finding of individual necrotic epithelial cells in the basal or suprabasal layers (180,181). These degenerating cells sometimes have closely adjoining lymphocytes forming the appearance of satellitosis (see below). Grade III involvement is characterized by intermittent regions of necrosis with bulla formation. Grade IV disease resembles toxic epidermal necrolysis. A morphometric study of skin has shown that rete ridges are the primary sites of attack in early GVHD (182). The concentration of stem cells in ridges for various anatomic sites was directly proportional to the frequency and concentration of GVHD lesions, implying that GVHD preferentially attacks less differentiated cells.

Histologically, hepatic GVHD is characterized by cholestatic hepatitis and degeneration or paucity of small bile ducts (179,183–185). Although hepatic GVHD may resemble viral hepatitis and drug-related injury, GVHD lesions may be distinguished by cytoplasmic eosinophilia of bile duct cells, segmental necrosis of bile ducts, cholestasis, and lobular disarray. Portal triads can be expanded by a primarily lymphocytic inflammatory infiltrate. The grading system originally proposed by Lerner *et al.* (179), based on the proportion of affected bile ducts, has fallen into disfavor because interpreting reactive versus degenerative changes can be difficult and because changes may be more affected by the duration of disease than by its severity.

In the gastrointestinal tract, individual crypt cell necrosis and lymphocytic infiltration unaccompanied by widespread inflammation or other cell destruction represent the hallmark findings of acute GVHD (179,186). These are most evident in rectal biopsies. Changes are localized to the bases of crypts in mild GVHD and extend to the surface epithelium in more severe disease. Increasingly severe disease is characterized by formation of crypt abscesses and crypt dropout, leading to epithelial denudation.

Ultrastructural studies of GVHD lesions have implicated lymphocytes as effectors that damage epithelial cells in the skin, liver, and gastrointestinal tract (187–189). Lymphocytes extend broad pseudopods with point contacts that indent but do not breach the cytoplasmic membranes of individual epithelial cells which often appear to be degenerating or necrotic. The point contacts between lymphocytes may represent the recognition phase of cell-mediated cytolysis. Immunohistologic studies have identified cells with markers of cytotoxic/suppressor T cells (CD8), helper T cells (CD4), NK cells (HNK-1), and Ia-positive lymphocytes infiltrating skin or gut in human GVHD (190–194a). The cells in close contact with basal keratinocytes most often appeared to be CD8 positive, consistent with the hypothe-

sis that they may have cytotoxic function. In a murine model of GVHD caused by minor antigen disparity, Ferrara *et al.* (139) found that the cells in close contact with keratinocytes had a surface phenotype characteristic of NK cells (Thy-1$^+$, Mac-1$^+$, asialo GM1$^+$, Ia$^-$, Lyt-1$^-$, Lyt-2$^-$). These findings were consistent with observations that administration of an anti-asialo GM1 antibody can prevent GVHD in irradiated mice given marrow and spleen cells from minor antigen histoincompatible donors (195).

On the other hand, peripheral blood T cells with cytotoxic specificity for host skin fibroblasts can frequently be recovered from patients with acute GVHD for more than 30 days, but are seldom recovered from patients without acute GVHD or from patients with acute GVHD for less than 30 days (196). Certain cells recovered from blood or involved skin of patients with acute GVHD have MHC-restricted recognition of minor histocompatibility antigens in proliferative or cytotoxicity assays (197,198). These studies are consistent with findings of Korngold and Sprent that MHC-restricted cells recognizing minor histocompatibility are required for the initiation of GVHD (199,200). However, MHC-restricted cells recognizing minor histocompatibility antigens were also detected in a patient who did not have acute GVHD (201) and Hamilton (202) was unable to show consistent correlation between cytolytic T lymphocytes and lethal GVHD in mice. The extent to which T cell-specific recognition or NK activation account for the effector phase of acute GVHD and the possible role of other mechanisms such as delayed type hypersensitivity (202a,b) remain unresolved.

3. *Prognostic Factors*

Certain prognostic factors have been related to the development of acute GVHD after allogeneic bone marrow transplantation. HLA disparity is a clear risk factor for acute GVHD (14). In leukemic patients given methotrexate for GVHD prophylaxis, the cumulative incidence of clinically significant grades II through IV acute GVHD was 42% in patients receiving HLA matched transplants from HLA genotypically identical siblings and 70% in patients receiving HLA-mismatched transplants from haploidentical relatives incompatible for antigens of one, two, or three HLA loci of the nonshared haplotype. Furthermore, the onset of disease occurred earlier in patients receiving mismatched transplants (median day 14) than in patients receiving matched transplants (median day 22). Disparity for HLA class I A-locus or B-locus antigens and disparity for class II HLA-D locus antigens had similar effects on GVHD risk. Patients receiving HLA phe-

notypically identical marrow had a 34% cumulative incidence of acute GVHD, similar to that of patients receiving HLA genotypically identical marrow. These results clearly emphasize the importance of MHC antigens on risk of acute GVHD after allogeneic bone marrow transplantation, confirming earlier findings in animal models.

A striking effect of patient age on the risk of acute GVHD has been observed in many studies (14,203–206), but the reason for increased risk in older patients is not readily explained. A possible role for reduced thymic function in older patients is suspected. This hypothesis is consistent with the concept that T cells newly generated in the recipient can contribute to acute GVHD and implies that a functioning thymus can prevent potentially alloreactive cells from reaching the periphery. In this regard, it is of interest that an autoimmune syndrome similar to GVHD has been observed in neonatally thymectomized mice (207,208).

Maintenance in a germ-free environment has been observed to reduce GVHD-related mortality in certain murine marrow transplant models (209–213). Mechanisms for this effect are unknown, but may be related to fortuitous cross-reactivity between bacterial antigens and host histocompatibility antigens or to nonspecific effects of lymphocyte activation by bacterial flora. Some clinical studies have shown that patients transplanted in a germ-free environment also have a reduced incidence of acute GVHD (206). This effect was most striking for patients with aplastic anemia who received methotrexate prophylaxis and were kept in isolation for 50 days. An effect has not been observed in leukemic patients maintained in germ-free isolation for shorter periods or in aplastic patients receiving improved GVHD prophylaxis with cyclosporine plus methotrexate. Gut decontamination with oral nonabsorbable antibiotics may be less effective in leukemic patients because of noncompliance related to nausea and vomiting.

Several studies have shown that cytotoxic pretreatment of the recipient increases the severity of GVHD in F_1 mice given parental marrow (214–217). Subclinical injury caused by cytotoxic therapy may exacerbate the manifestations of GVHD. Alternatively, the injured cells may have an enhanced ability to stimulate an anti-host response. Finally, cytotoxic pretreatment may overcome elements of resistance, allowing more vigorous engraftment of GVHD effector cells. One study has shown that GVHD in mice given minor antigen histoincompatible marrow can be exacerbated by transfusion of irradiated MHC-incompatible spleen cells (218). Transfusion might stimulate the generation of effectors specific for minor histocompatibility antigens of the recipi-

ent or might induce production of lymphokines which exacerbate GVHD.

Hill *et al.* (88) found that aplastic patients with complete chimerism had a 57% cumulative incidence of acute GVHD compared to only 18% in the mixed chimeras. The mechanisms leading to mixed chimerism and avoidance of acute GVHD in aplastic patients are conjectural. Donor cells with histocompatibility antigens more closely matched with those of the recipient may have a simultaneously reduced ability to eradicate residual host cells and cause GVHD. Persisting host cells may also actively suppress or eliminate alloreactive donor cells.

An effect of sex mismatching between donor and recipient has been observed in a number of studies. Results have been somewhat inconsistent with some studies showing increased risk of GVHD in sex-mismatched recipients of either sex (205,206) and other studies showing increased risk only in male recipients of female marrow (203,204). Recent data from the International Bone Marrow Transplant Registry (M. Bortin, personal communication) and an Australian study (218a) have indicated that GVHD is particularly increased in recipients given marrow from previously pregnant women, implying a role for alloimmunization against minor histocompatibility antigens.

The incidence of GVHD in patients with aplastic anemia transplanted from HLA-identical donors appears to be affected by certain HLA-B antigens (219). Patients with HLA-B18 had a risk of GVHD nearly threefold higher than other patients, and patients with HLA-B8 or HLA-B35 had only half the risk of GVHD compared to other patients. Association with HLA-A antigens was not detected. These findings might reflect linkage disequilibrium between certain HLA-B antigens and immune response genes responsible for acute GVHD.

Vogelsang *et al.* (220) have developed a test that could serve as a prognostic indicator of acute GVHD for individual donor–recipient pairs. Recipient skin explants were cocultured with donor lymphocytes previously stimulated by recipient cells in MLC. Sections were then examined for histologic evidence of acute GVHD. In patients receiving HLA-identical marrow and cyclosporine or cyclophosphamide prophylaxis, the test had a sensitivity of 84% and a specificity of 85% for predicting the histologic occurrence of grades II through IV acute GVHD with similar results for predicting clinical severity. It was noteworthy that this test could detect alloreactivity, whereas other conventional tests such as proliferation or generation of cytotoxic T cells in mixed lymphocyte culture could not. Alloreactivity between HLA-identical siblings can be detected by proliferative re-

sponses in mixed epidermal cell-lymphocyte culture (220a). This test may also be useful for predicting GVHD.

4. Treatment of Acute GVHD

Treatment of established acute GVHD poses a difficult problem. Patients with untreated GVHD are at high risk of developing viral, bacterial, and fungal infections for at least three possible reasons. First, lesions in the skin and gastrointestinal tract allow entry for infectious agents. Second, acute GVHD may compromise the already impaired immune function of transplant recipients (see below). Third, GVHD may reactivate latent cytomegalovirus or herpes simplex virus (221–223). On the other hand, available modes of treatment for acute GVHD involve the use of nonspecific immunosuppressive agents, which in turn can predispose toward infectious complications. The dilemma, then, is how to provide sufficient immunosuppression for control of GVHD without excessive immunosuppression that would lead to infectious complications.

Corticosteroids (224–228), cyclosporine (229,230), ATG (57), and murine monoclonal antibodies (231,232) have been used for treatment of acute GVHD. In general, partial or complete responses have been observed in 40–60% of patients treated with single-agent corticosteroids, cyclosporine, or ATG. Similar results have been observed with the combined use of ATG and cyclosporine (233). Use of all three agents did not improve the response rates and was in fact associated with a decreased survival because of an increased incidence of interstitial pneumonia. A high proportion of patients who survive acute GVHD subsequently develop chronic GVHD, such that only 25% of patients survive beyond 17 months (234). Experience with monoclonal antibodies for treatment of acute GVHD is limited although preliminary results suggest that treatment with certain anti-CD3 (232) or anti-CD6 (234) antibodies are active in suppressing disease activity. Patients who received an anti-CD3 antibody responded only as long as antibody treatment was continued, and some developed a fatal Epstein–Barr virus-associated polyclonal proliferation of donor-derived B lymphocytes. It was suggested that the combined effect of severe end-stage GVHD and potent immunosuppressive therapy with an anti-T cell-specific antibody resulted in a progressive immunodeficiency syndrome. This in turn might have abrogated the T cell-mediated surveillance mechanism that normally modulates the proliferation of EBV-infected lymphocytes.

A new approach to the treatment of acute GVHD has been suggested by Vogelsang *et al.* (234a) who found that administration of

thalidomide could resolve all manifestations of GVHD in irradiated rats given MHC-incompatible marrow. Importantly, there was no flare of disease activity after stopping the treatment. The results suggested that thalidomide may act by facilitating the development of cells capable of suppressing anti-host activity, resulting in specific tolerance. In the same study, thalidomide was also found to be useful for preventing GVHD.

5. *Prevention of Acute GVHD*

Methotrexate was first developed as an agent for prophylaxis of GVHD after allogeneic marrow transplantation in murine (235) and canine (236,237) models. The dose schedule conventionally used in humans is 15 mg/m^2 on day 1, and 10 mg/m^2 on days 3, 6, 11, and weekly thereafter until day 100. Because of convincing data in animal models, randomized studies comparing the use of methotrexate versus no posttransplant immunosuppression have never been carried out in humans. However, two nonrandomized studies have recently addressed the need for posttransplant immunosuppression in humans. In leukemic patients prepared with cytosine arabinoside and TBI, Lazarus *et al.* (238) found a high incidence (70%) of acute GVHD whether methotrexate was given or not. Sullivan *et al.* (239) subsequently found that all 15 recipients of HLA-matched marrow not given posttransplant immunosuppression developed moderate to severe acute GVHD that was early in onset (median day 8). In a corresponding group of comparable patients who received methotrexate, the cumulative incidence of clinically significant acute GVHD was 25%, in agreement with findings of other studies. Reasons for the high incidence of acute GVHD in methotrexate-treated patients in the earlier study are not clear, but may be related to patient selection, the pretransplant conditioning regimens, or small sample size. A beneficial effect of methotrexate was also indicated in one randomized prospective study (240) which showed that patients who received only the first four doses of methotrexate had a higher incidence of acute GVHD than patients who received methotrexate according to the conventional schedule (see below).

Cyclosporine is a noncytotoxic and nonmyelosuppressive agent recently introduced for prevention of acute GVHD. This immunosuppressive drug is capable of inhibiting production of IL-2, expression of IL-2 receptors, T cell proliferation, and differentiation of precursor cytotoxic T lymphocytes [see (241) for review]. A large number of recent clinical studies have evaluated the use of cyclosporine for prophylaxis of GVHD (242–246). Patients receiving cyclosporine and pa-

tients receiving methotrexate have a similar incidence of acute GVHD and similar survival.

A regimen which combines cyclosporine with four doses of methotrexate administered on days 1, 3, 6, and 11 has been evaluated in humans, based on preclinical studies by Deeg *et al.* (247) demonstrating improved survival in a canine model. Two randomized prospective studies, one in patients with acute nonlymphocytic leukemia in first remission or chronic myelogenous leukemia in chronic phase (248) and the other in patients with aplastic anemia (249), have shown a reduced incidence of acute GVHD when combined cyclosporine and methotrexate were used, compared to either cyclosporine or methotrexate alone. More importantly, survival of patients who received combined prophylaxis was improved in both studies, indicating that this regimen was capable of reducing GVHD risk without increasing toxicity or producing significant immunodeficiency. In fact, the frequency of deaths from infections associated with GVHD was reduced in patients receiving the combined regimen, whereas the frequency of deaths from infections not associated with GVHD was similar in the two groups of both studies. An unexpected finding in both studies was that the survival benefit appeared to be most pronounced in the period after the first 100 days, despite the fact that the incidence of chronic GVHD was similar in the two groups of both studies. Some of the patients had a protracted course of acute GVHD. In addition, patients developing chronic GVHD after acute GVHD are known to have more fatal infectious complications than patients developing *de novo* chronic GVHD.

Prevention of acute GVHD by depletion of T cells or lymphocytes in donor marrow has been demonstrated in numerous rodent models (166–169) as well as in canine (250) and nonhuman primate models (251). These results have prompted a number of different clinical studies exploring the feasibility of preventing acute GVHD by T cell depletion through a variety of methods including lectin agglutination with E rosetting (133,252,253), elutriation (254,255), monoclonal antibodies and complement (124,127,128,135,256,257), or toxin-linked monoclonal antibodies (258). For patients receiving HLA-identical marrow, results in nearly all studies have demonstrated a significant reduction in acute GVHD if at least a 1.5 log T cell depletion is achieved, regardless of the method used. A preliminary compilation of results from several centers showed that only 7% of patients with durable grafts had clinically significant acute GVHD after receiving T cell-depleted HLA-identical allogeneic marrow and no posttransplant immunosuppression (259). Even with adjustments for patient age and

early death, these data provide convincing evidence for a favorable effect of T cell depletion on GVHD. As described above, previous studies have shown that omission of immunosuppression after transplantation of HLA-identical unmodified marrow is associated with moderate to severe GVHD in most recipients.

The mechanisms by which T cell depletion can prevent acute GVHD may not be entirely straightforward in view of observations that graft failure and mixed chimerism are increased in patients receiving T cell-depleted allogeneic marrow. If graft enhancement and GVHD represent the effects of distinct T cell populations, depletion of either population could prevent GVHD. Depletion of the graft-enhancing population could prevent engraftment of GVHD-producing cells. In this case, GVHD might occur if the marrow contained GVHD-producing cells and if engraftment could be achieved by some other means such as increased pretransplant immunosuppression. Depletion of GVHD-producing cells would prevent GVHD under any circumstance. On the other hand, if graft enhancement and GVHD represent effects of a single T cell population, then sufficient depletion of these cells would always prevent GVHD, even if engraftment could be achieved in the absence of a graft-enhancing effect. For these reasons, it becomes essential to document chimerism of both hematopoietic cells and lymphocytes, preferably at frequent intervals, in all patients receiving T cell-depleted allogeneic marrow. In patients not given posttransplant immunosuppression, the absence of GVHD in the setting of mixed chimerism could reflect either the absence of GVHD-producing cells in the graft or the inability of these cells to become engrafted, whereas the absence of GVHD in the setting of full chimerism unambiguously indicates that GVHD-producing cells were absent from the graft.

The extent of T cell depletion required to prevent GVHD is unknown and probably depends on multiple other factors, including recipient age, genetic disparity between donor and recipient, conditioning regimen, posttransplant immunosuppression, and possibly the type of reverse isolation. Observations in animal models have indicated that for greater degrees of genetic disparity, fewer T cells are required to cause acute GVHD. A smaller number of residual T cells may be sufficient to produce GVHD following immunosuppressive conditioning regimens that reliably produce full chimerism as opposed to regimens associated with frequent mixed chimerism or graft failure. Likewise, larger numbers of T cells may be tolerated in patients receiving posttransplant immunosuppression. Finally, it is possible that GVHD might occur in patients receiving marrow totally

devoid of all mature T cells if alloreactive (or possibly autoreactive) cells can be newly generated in the host after transplantation. Because of the complex multivariable nature of GVHD risk, results may differ among studies. Indeed, we have seen grade II GVHD occurring in one patient who received only 1.7×10^3 T cells/kg (1.2×10^5 total T cells) and grade III GVHD occurring in another patient who received only 4×10^3 T cells/kg (2.7×10^5 total T cells). These results contrast with those of other investigators who report little or no GVHD in patients receiving at least 10 times as many T cells, and no posttransplant immunosuppression (135,256,257,260,260a).

C. Chronic Graft-versus-Host Disease

1. Clinical Manifestations

Chronic GVHD has been described as a syndrome with a spectrum of clinical manifestations resembling several naturally occurring collagen vascular diseases (261–264). Initial manifestations of the disease generally appear after day 100 but can appear as early as day 70. In most patients the disease follows acute GVHD either in direct progression or with an intervening quiescent period. Twenty to 30% of patients with chronic GVHD have a *de novo* late onset without prior acute GVHD. Symptoms of chronic GVHD resemble those of Sjogren's syndrome, polymyositis, lichen planus, scleroderma, and primary biliary sclerosis with involvement of the skin, liver, mouth, eyes, and esophagus.

The manifestions of chronic GVHD of the skin differ from those of acute GVHD, and often occur in sun-exposed areas with a characteristic progression from erythema or insidious pigmentary changes to lichen planus, dermal thickening, epidermal atrophy, and sclerosis (265–269). Hepatic abnormalities resemble those of chronic active hepatitis and cholestasis, generally not accompanied by signs of portal hypertension and cirrhosis. Oral involvement develops as xerostomia, mucosal atrophy, and lichenoid reactions on the buccal mucosa and tongue (270,271). Salivary changes include decreased IgA, and increased sodium, albumin, and IgG concentrations (271–273). Xerostomia can lead to severe dental caries and periodontitis. Eye disease initially manifests itself as a symptomatic sicca and can progress from xerophthalmia to keratoconjunctivitis sicca (274). Esophageal involvement can present as stricture formation or motility disorders (275,276). Recurrent bacterial infections, malabsorption, weight loss, and contractures can occur in untreated patients.

Clinically, chronic GVHD has been graded in "limited" and "extensive" categories (263). Limited disease indicates localized skin involvement with or without abnormal liver function tests due to chronic GVHD. Extensive disease indicates either generalized skin involvement or localized skin involvement with chronic aggressive hepatitis, bridging necrosis or cirrhosis on liver biopsy, or involvement of any other target organ. Asymptomatic patients with changes on skin and oral biopsies on day 80 after transplantation have been defined as having "subclinical disease." These patients frequently develop subsequent overt clinical disease, whereas patients with normal biopsies do not. Overall, approximately 30 to 50% of patients surviving for more than 150 days after transplantation develop chronic GVHD. Patients with limited disease fare better than patients with extensive disease, and patients with *de novo* onset fare better than patients with antecedent acute GVHD.

2. *Pathologic Manifestations*

The pathologic manifestations of chronic GVHD are pleomorphic, depending on the stage of the disease. The skin shows changes in all layers (268). In the early phase of generalized chronic GVHD, epidermal hypertrophy and hyperkeratosis are accompanied by eosinophilic body formation and lichenoid reactions in the basal layers, thickening of the basement membrane zone, edema of the papillary dermis, and inflammatory infiltration of the pilar units, eccrine coils, and subcutaneous fat. Immunohistologic studies show deposits of immunoglobulin and complement at the dermoepidermal junction. In advanced disease, these changes are replaced by epidermal atrophy, obliteration of rete ridges and the dermoepidermal junction, and dense fibrosis throughout the dermis and adnexal structures. Changes similar to those of advanced disease appear to develop directly in localized lesions of chronic GVHD.

The histologic findings in chronic GVHD of the liver are similar to those of acute GVHD and depend mostly on the duration of active disease (262). Findings in long-standing disease are variable and may show inflammatory piecemeal necrosis or bridging necrosis, sometimes leading to fibrosis, but always accompanied by cholestasis and paucity of small bile ducts. The squamous oral mucosa, salivary ducts, conjunctival epithelium, and lacrimal ducts show mononuclear infiltration with epithelial cell necrosis, destruction of glands and ducts, and interstitial fibrosis (277). The esophagus shows mononuclear cell inflammation, epithelial necrosis, and submucosal fibrosis. Thymic

atrophy and fibrosis have also been found at autopsy in patients with chronic GVHD (262).

3. *Cellular Mechanisms in Chronic GVHD*

Patients with chronic GVHD develop a severe immunodeficiency not found in long-term healthy marrow transplant recipients (see below). Experiments by Tsoi *et al.* (278–280) have suggested that suppressor cells could contribute to the immunodeficiency of chronic GVHD. Unirradiated cells from healthy long-term marrow transplant recipients and from patients with chronic GVHD were tested for their ability to inhibit the mixed lymphocyte response of donor cells stimulated by irradiated third-party peripheral mononuclear cells. The presence of such suppressor cells was strongly correlated with chronic GVHD. Sequential studies showed that nonspecific suppression was not present in most patients before the development of active chronic GVHD and that suppression tended to disappear as the disease resolved. Further studies indicated that nonspecific suppression was mediated by radiation-sensitive (16.0 Gy) nonadherent CD8-positive cells. Similar findings correlating nonspecific suppressor activity with chronic GVHD have been described in a rat model (281,282).

Other experiments by Tsoi *et al.* (283) suggested that tolerance after allogeneic marrow grafting is maintained by a specific suppressor cell mechanism. Unirradiated cells from healthy long-term marrow transplant recipients and from patients with chronic GVHD were tested for the ability to inhibit the mixed lymphocyte response of donor cells stimulated by irradiated trinitrophenol (TNP)-modified cryopreserved pretransplant peripheral blood cells of the host. TNP was used in order to generate proliferative responses between HLA-identical donor and recipient cells. The premise underlying the study was that TNP-modified non-MHC polymorphic antigens could be recognized in a primary mixed lymphocyte reaction and that recognition of these antigens might be controlled by suppressor cells. Unmodified cells from healthy long-term chimeras suppressed the response of donor cells stimulated by TNP-modified host cells but did not suppress the response to TNP-modified donor cells, TNP-modified third party cells, or unmodified third party cells. Cells from patients with chronic GVHD generally did not suppress the response of donor cells stimulated by TNP-modified host cells but often suppressed the response to unmodified third party cells. Peripheral blood cells from patients with chronic GVHD sometimes show one-way proliferative responses after stimulation in mixed lymphocyte culture with cryopreserved host cells (280,284). This finding was significantly less common when cells

from healthy long-term chimeras were tested. Thus cells from patients with chronic GVHD show a constellation of possibly interrelated findings including nonspecific suppression, absence of donor-specific suppression, and proliferative responsiveness to stimulation by host cells.

The mechanisms of nonspecific and specific suppression described above have not been elucidated. It is possible that nonspecific suppression could be overcome by exogenous IL-2, as has been described for concanavalin A-generated suppressor cells (285). It is attractive to speculate that veto cell dysfunction might account for some of the findings in chronic GVHD, since veto cells have been postulated to prevent the development of autoimmunity. The specific suppressor cells found in healthy long-term chimeras, however, do not fulfill the criteria of veto cells described by Miller (154), who found that unmodified cells were not able to suppress responses against TNP-modified cells.

4. Risk Factors for Chronic GVHD

Acute GVHD represents the major risk factor for development of chronic GVHD (263). Cytotoxic cells involved in acute GVHD may release lymphokines that stimulate expression of class II antigens on epithelial cells. This in turn may provoke T cells to secrete lymphokines that promote collagen synthesis (177b). Alternatively, acute GVHD may damage the immune system, and particularly the thymus, which in turn may predispose to immune dysfunction and development of chronic GVHD. Support for this hypothesis comes from the observation that in patients without acute GVHD the incidence of chronic GVHD increases with age (263,286,287). Thymic hypofunction caused by age-related involution or by damage from acute GVHD may permit the generation of nontolerant T cells which in turn can cause chronic GVHD.

The different effects of mixed chimerism and buffy coat infusions suggest that acute and chronic GVHD have distinct pathogenic mechanisms. Mixed chimerism was not associated with reduced chronic GVHD in aplastic patients even though acute GVHD was reduced (88). Although buffy coat infusions had no effect on acute GVHD in aplastic patients, there was a marked increase in *de novo* onset chronic GVHD (81,288). Buffy coat transfusions may induce a qualitative change that predisposes to the development of chronic GVHD. Alternatively, the increased chronic GVHD may be related to a dose–response effect in donor–recipient pairs having minimal genetic disparity. It is noteworthy that the buffy coat cells did have a graft-

enhancing effect despite the fact that they did not exacerbate acute GVHD in aplastic patients. As noted below, however, buffy coat infusions did exacerbate acute GVHD in leukemic patients.

5. *Treatment and Prevention of Chronic GVHD*

Preliminary trials suggested that combination therapy with prednisone and either azathioprine, cyclophosphamide, or procarbazine could achieve better survival than treatment with prednisone alone (263). A subsequent randomized trial comparing prednisone plus azathioprine versus prednisone plus placebo has shown less frequent development of contractures but increased infections and decreased survival in patients receiving combination therapy (Sullivan *et al.*, unpublished). Improved nutritional support, routine antibiotic prophylaxis, and earlier initiation of therapy may have decreased disability due to progressive disease and improved survival compared to the earlier studies. Current trials are evaluating daily alternating therapy with prednisone and cyclosporine.

Few studies have been deliberately designed for the prevention of chronic GVHD. Early uncontrolled trials of long-term corticosteroid therapy have shown inconsistent results. The recent studies of cyclosporine plus methotrexate for prophylaxis of acute GVHD have shown no effect on chronic GVHD, possibly because some of the patients receiving single-agent prophylaxis developed acute GVHD and died before chronic GVHD could develop (248,249). Subclinical GVHD and related pathological changes predisposing to chronic GVHD might persist despite increased immunosuppressive treatment. Some of the recent studies using T cell-depleted marrow have suggested a decreased incidence of chronic GVHD. Preliminary analysis of Seattle data has shown that only 3 of 27 evaluable patients transplanted with T cell-depleted marrow developed chronic GVHD of severity sufficient to require treatment (unpublished). This result will require confirmation in populations where survival would allow evaluation of a larger proportion of patients.

IV. Immune Reconstitution

The chemotherapy and TBI given in preparation for marrow transplantation cause extensive ablation of cellular and humoral immunity. All patients develop a transient but profound immunodeficiency. In fact, graft rejection and persistence of isohemagglutinins are among the few clinical manifestations that represent vestiges of host immunity after marrow transplantation. With time, however, donor-derived

lymphoid cells repopulate the host, ultimately resulting in complete restoration of immunocompetence. Studies of informative markers after marrow transplantation have established the donor origin of blood and marrow T lymphocytes, blood B lymphocytes, alveolar and hepatic macrophages, and serum immunoglobulin (63,65,288–294). The time for restoration varies for different components and functions of the immune system, but the general pattern appears little affected by the underlying disease, conditioning regimen, allogeneic or syngeneic source of the marrow, or type of posttransplant immunosuppression (295–297). Other than time, GVHD represents the only factor having a major impact on immune reconstitution after marrow transplantation, although a possible role for HLA identity has been suggested in one study were an increased risk of late nonvaricella zoster infections was found in patients who received MHC partially incompatible marrow (298). Attempts to accelerate immune reconstitution by transplantation of thymic tissue or by treatment with thymosin fraction 5 or thymopoietin pentapeptide have been unsuccessful (299,300).

A. Repopulation of Lymph Nodes and Thymus

Few clinical studies have addressed repopulation of lymph nodes and thymus after marrow transplantation. An autopsy series studied by Drenguis and Sale (301,302) showed that lymph node cortical cellularity was moderately decreased for the first 70 days and thereafter increased gradually toward normal. It was of interest that recipients of syngeneic grafts showed a more striking decrease in cellularity during the first 30 days than allogeneic grafts, suggesting that a lymphoid proliferation phase may occur in the latter. Poorly defined round nodules resembling primary follicles were identified in most patients, but formation of well-defined secondary germinal centers was unusual before day 100. Immunoglobulin-positive cells usually could not be identified in specimens obtained before day 14. Thereafter IgM- and IgG-positive cells were uniformly seen in lymph nodes from syngeneic recipients but sometimes not in allogeneic recipients. There appeared to be a slight predominance of IgM-positive cells before day 50, but there was no evidence of clonal restriction.

The thymus has been difficult to study in human transplant recipients due to the variable effects of age-related involution, stress, viral infection, corticosteroids, and GVHD. In one series, all specimens showed severe cortical involution (303). Before 2 months, the medulla and perivascular spaces were lymphocyte depleted. Thereafter, mature small lymphocytes were present primarily in the perivascular

spaces, but lymphoblasts and immature medium-sized lymphocytes were not seen for up to 123 days after transplantation. Cellular degeneration and plasma cell infiltration were present in samples from patients affected by GVHD or disseminated infections. On the other hand, the thymus of a 13-year-old boy killed in an automobile accident more than 5 years after receiving an allogeneic marrow transplant showed completely normal histology (302).

B. Phenotype and Function of Peripheral T Cells

During the first 2 weeks after marrow transplantation there are almost no circulating lymphocytes. With engraftment, lymphocytes gradually increase in numbers, reaching normal levels by 2 to 3 months (304,305). Cell surface phenotyping has uniformly shown that the number of Ia-positive cells is increased and that there is an abnormal preponderance of CD8-positive cells compared to CD4-positive cells (306–314). Patients with GVHD appear to have delayed regeneration of CD4-positive cells. The number of "OKT-10" positive cells is increased in all patients regardless of GVHD, but these cells do not express transferrin receptors, and they disappear between 6 and 12 months after transplantation. CD1-positive cells expressing thymocyte-associated TLA-like antigens have not been consistently identified.

One study showed that T cells from marrow transplant recipients have some phenotypic characteristics that resemble those of thymocytes (315). Lymphocytes from patients showed binding of peanut lectin, had a thymocyte-like pattern of LDH isoenzymes, and had low activities of ecto-5′-nucleotidase and purine nucleoside phosphorylase. Resolution of these abnormalities correlated closely with reconstitution of proliferative responses to mitogens and allogeneic cells. It could not be resolved whether these findings represent an expansion of cells normally found in small numbers in healthy individuals or whether they represent an abnormal population of immature cells.

Proliferative responses to mitogens, antigens, and allogeneic cells are low for up to 6 months after marrow transplantation, and then return to normal, except in patients who had GVHD (304,305,316,317). There is some evidence that suppressor cells may be responsible for the low proliferative responses in mixed lymphocyte culture (317a). On the other hand, a study by Welte *et al.* (318) showed that lymphocytes from marrow transplant recipients have defective IL-2 production after stimulation with PHA or with anti-CD3 antibody. IL-2 could not be detected in culture supernatants of PHA or CD3 antibody-stimulated cells. Responses to CD3 antibody were

completely restored by adding highly purified IL-2 to the cultures, and responses to PHA were partially restored. Thus, activated cells could express IL-2 receptors but could not secrete IL-2. These characteristics are similar to those of thymocytes stimulated by anti-CD3 antibodies (319). Brkic *et al.* (320) showed that the ability to produce IL-2 in response to PHA stimulation returned to normal by 1 year after transplantation in recipients without chronic GVHD, but remained abnormal in patients with chronic GVHD.

Further studies showed that cytotoxic T lymphocytes could be generated by stimulating patient cells in mixed lymphocyte cultures as early as day 54 after transplantation provided the patient had no GVHD (321). Generation of cytotoxic T lymphocytes with cells from patients with acute GVHD could be restored by adding exogenous IL-2 to the cultures, suggesting insufficient IL-2 production. Exogenous IL-2 did not enable cytotoxic T lymphocytes to be generated with cells from patients with chronic GVHD, possibly because the precursors lack receptors for IL-2 or because of nonspecific suppression. A noteworthy observation in this context is that the ability to generate virus-specific cytotoxic T lymphocytes is highly correlated with survival in patients after CMV infection (322).

Slightly different results have been reported when limiting dilution methods were used to enumerate mitogen-reactive precursors of cells capable of secreting IL-2 and cells capable of cytotoxic function (322a). The precursor frequencies for these cells remained low for as long as 6 years after transplantation, whereas the precursor frequency for cells capable of proliferating in response to PHA often reached normal values within 1 year. The possible role of GVHD in causing abnormally low helper and cytotoxic precursor frequencies has not yet been analyzed.

A paradoxical result was found when testing cutaneous contact sensitivity to dinitrochlorobenzene (DNCB) and delayed type hypersensitivity responses in marrow transplant recipients (323). Approximately 75% of patients were able to respond to DNCB at 100 days after transplantation. By 150 days, the proportion of responding patients decreased to 35%, and subsequently increased again to 75% at 2 years, and 92% at 4 years. It has been speculated that a persisting population of host cells might account for the reactivity at day 100. The response rate was not affected by acute or chronic GVHD, but was decreased in patients receiving cyclosporine. Approximately 60 to 75% of patients without acute GVHD were able to respond to one or more recall antigens when tested between 100 days and 3 years after transplantation. Only 25 to 50% of patients who had moderate to se-

vere GVHD showed responses. By 4 years, 11 out of 12 patients tested were able to respond.

C. Humoral Immunity

Serum immunoglobulin levels decrease after marrow transplantation and return to normal range at 9 to 14 months for IgM and IgG, and at 2 years for IgA in patients without GVHD (304,305,317). Patients who develop GVHD have increased levels of IgM between 4 and 8 months and increased IgG between 8 months and 3 years after transplantation. Most patients have a transient elevation of IgE levels beginning between 2 and 5 weeks after transplantation and lasting for 1 to 7 weeks (324–326). In studies by Ringden *et al.* (325), onset of the increase in IgE often appeared to coincide with development of acute GVHD. Studies with allotypic markers have shown that serum immunoglobulin can be completely donor derived as early as day 80 after transplantation. However, host-type isohemagglutinin can persist for up to 120 days (327), suggesting that immunoglobulin production by host cells may continue for some period after marrow transplantation. These findings are consistent with the known radioresistance of plasma cells.

Several studies now show that specific humoral immunity can be transferred from donor to recipient. One investigation demonstrated transfer of anti-KLH activity from an immunized donor to an unimmunized identical twin recipient (328). Another case report documented transfer of humoral immunity to acetylcholine receptors and development of myasthenia gravis in a previously unaffected recipient given marrow from an affected donor (329). Studies of donor–recipient pairs who were not recently boosted have shown transfer of immunity to tetanus toxoid, measles, and diphtheria from seropositive donors to seronegative recipients, and loss of immunity to measles and diphtheria in transplants from seronegative donors to seropositive recipients (330). Overall, titers at 100 days after transplantation were similar to pretransplant titers of either the donor or recipient, and could represent the combined effects of persisting host production, blood product transfusions, and production by precommitted donor memory B cells. Long-term transfer and persistence of humoral immunity was successful in 85% of healthy survivors but failed in 55% of patients with chronic GVHD. Cell separation studies have shown that both the T cell component and the B cell component of specific immunity to tetanus toxoid are transferred to recipients after marrow transplantation (331).

Repopulation of B lymphocytes in marrow transplant patients have shown some unusual patterns (332). Cells expressing surface immunoglobulin have been identified as early as 20 to 30 days after transplantation (317). One population of B cells had surface IgM and IgD together with HLD-DR and CD20. Another population was unusual in having higher amounts of IgM, lower amounts of IgD, and weak expression of CD5, a marker found on most T cells but also frequently found on chronic lymphocytic leukemia cells. Large numbers of similar cells have recently been identified in human fetal spleen, suggesting the hypothesis that they may represent an early stage of normal B cell differentiation. Alternatively, the CD5-positive B cells could represent the end products of a distinct lineage.

The development of humoral immune responsiveness has been directly tested by immunizing marrow graft recipients with neoantigens (327). Patients without GVHD showed normal primary and secondary responses to bacteriophage by 180 days, and normal secondary responses to keyhole limpet hemocyanin (KLH) by 100 to 300 days, depending on age. Responses to pneumococcal polysaccharide, however, did not become normal until 2 years after transplantation, and primary responses to KLH remained subnormal even after 3 years. Specific primary and secondary antibody responses to all exogenous neoantigens remained decreased in patients with chronic GVHD.

Studies have been carried out to characterize B lymphocyte function in marrow transplant recipients and to assess the impact of GVHD (333–340). During the first 3 months after transplantation, pokeweed mitogen (PWM)-stimulated B lymphocytes do not produce immunoglogulin even in the presence of normal T cell help. Cell-mixing experiments carried out in a PWM-driven Ig plaque-forming assay system showed a variety of abnormalities in isolated T cell subsets, including failure of helper function and inappropriate suppressor activity in CD4-positive cells (339,340). Abnormalities of T cells and B cells were frequent in the early posttransplant period, even in the absence of acute GVHD. Abnormalities of T cells and B cells were less frequent in cells from healthy long-term chimeras but often persistent in patients with chronic GVHD. In one study, B cell function appeared to return toward normal before T cell function (337). Similar results were found when studies of healthy long-term survivors and patients with chronic GVHD were carried out by using other T cell-dependent polyclonal B cell activators (341) such as herpes simplex virus (HSV) and tetanus toxoid (TT), as well as the T cell-independent B cell activator, Epstein–Barr virus (342). For any given patient, ab-

normalities in one assay system could not be generalized to other assay systems and could not be related to serum immunoglobulin levels, anti-HSV titers, and anti-TT titers. The profound inability to produce specific antibody in patients with chronic GVHD suggests a significant abnormality in cellular cooperation and may reflect a state of immune dysregulation.

Given the above information, it is striking that 80% of CMV-seronegative recipients of marrow from seronegative donors can show seroconversion after CMV infection, and that this response can occur as early as 36 days after transplantation (R. Bowden, unpublished). Thus, there is evidence that priming for the humoral immune response to herpes virus infection can occur in the early posttransplant period despite multiple abnormalities that have been described for *in vitro* B cell responses. The importance of the *in vivo* response is illustrated by the results of one study showing that among cases of interstitial pneumonia in which CMV was the only pathogen identified, none of the nine patients who did not seroconvert survived, whereas four out of five patients with seroconversion did (44).

D. Cytotoxic Effector Cells, Accessory Cells, and Neutrophils

Lymphocytes having surface antigen phenotypes characteristic of NK cells appear in strikingly elevated numbers during the first 90 days after transplantation (332). Cytotoxic effector cells mediating natural killing, antibody-dependent cellular cytotoxicity (ADCC), and lectin-dependent cellular cytotoxicity return to normal within 30 days after transplantation and remain normal for at least 100 days (343,344). Low activity was found, however, in approximately 20% of patients tested at 1 year. Low and normal or high cytotoxicity in one assay was not always accompanied by similar activity in the other two assays, unlike results with cells from healthy individuals. These findings suggest either that different cell populations or, more likely, that different cellular mechanisms are involved.

An association between pretransplant NK activity and GVHD has been found when cells from the recipient were tested against HSV-1-infected fibroblast target cells (345), but not when tested against K562 cells (340). Other studies have found that the highest levels of NK activity occurred during episodes of acute GVHD (342,346), and that decreased NK activity was associated with CMV infection (343). The level of NK cytotoxic activity was not associated with leukemia recurrence, suggesting that NK activity might not represent an important surveillance mechanism against leukemia *in vivo*. There are, how-

ever, a number of other possible explanations for the findings. Cells capable of controlling leukemia *in vivo* and cells capable of lysing K562 cells *in vitro* might not represent the same population. Second, the burden of leukemic cells remaining after transplantation might be too high to be controlled by donor NK cells. Finally, leukemias at an advanced stage of development might already be selected for NK resistance.

Blood macrophages are known to be almost entirely of donor origin as early as 41 days after transplantation (347), and uniformly have normal functional capabilities. Peripheral blood macrophages pulsed with heat-killed *Escherichia coli* had normal ability to induce T cell proliferation (347). Similarly, blood monocytes had normal accessory function for pokeweed-induced immunoglobulin production as early as 30 days after transplantation, and lipopolysaccharide-induced production of IL-1 was normal (320,348). Y-chromatin staining of cells obtained from liver or lung biopsy touch preps or from bronchoalveolar lavage provided evidence for the donor origin of hepatic Kupffer cells and alveolar macrophages in human marrow transplants (291,292,349). In the lung, the proportion of residual host macrophages showed a linear decay, indicating that, under these conditions, alveolar macrophages have a lifespan of approximately 81 days (291). Further studies have demonstrated that macrophages obtained by bronchoalveolar lavage during the first 4 months have defective function, as measured by chemotaxis, phagocytosis, and microbicidal activity (349). By 6 months, however, macrophage functions returned to normal except for a persistent defect in killing *Candida pseudotropicalis*.

The number of polymorphonuclear cells begins to approach normal levels within 3–4 weeks in most marrow transplant recipients. Neutrophil random migration, microbicidal activity, and iodination appear to be normal even early after transplantation (350–352). Neutrophil chemotaxis may be impaired during the first 4 months after transplantation, although conflicting results have been reported (350,351). Acute and chronic GVHD, ATG therapy, and infection itself may also produce subtle impairment of neutrophil chemotaxis.

E. Effects of T Cell Depletion

The ability to transfer specific immunity between donor and recipient suggests that immune reconstitution is at least partly determined by mature cells within the graft. On the other hand, there is evidence that responses to some antigens required induction of primary immu-

nity in the nascent posttransplant immune system. More rapid and frequent recurrences were observed in clinical trials where acyclovir was used to treat HSV infection (353,354). Serial determinations of HSV-specific lymphocyte transformation showed a delay in reconstitution of this response in patients receiving acyclovir compared to those receiving no treatment (354). Further studies showed that suppressor cells cannot account for the lack of response to HSV in the early posttransplant period (355). The cells responding to viral infection might well be immature lymphocytes generated from marrow stem cell precursors possibly processed in the host thymus after transplantation.

There is a theoretical concern that depletion of mature T cells from the donor marrow might be associated with increased immunodeficiency and infections after transplantation. Preliminary studies have shown that removal of T cells in the graft generally has little impact on the pattern or kinetics of T cell repopulation (332), although one study has suggested that T cell depletion with a mixture of anti-CD8 and anti-CD6 antibodies may prevent the high rebound of $CD8^+$ cells often seen after marrow transplantation (260). Another study showed that regeneration of NK activity was not affected by depletion of $CD8^+$ and $CD6^+$ cells in the donor marrow (356). In further studies, we have found that 20 out of 24 recipients of T cell-depleted marrow showed cutaneous responses to DNCB and 17 of 26 patients were able to respond to 1 or more recall antigens on day 100 after transplantation. Furthermore, one CMV-infected seronegative recipient of T cell-depleted marrow from a seronegative donor showed seroconversion on day 79 after transplantation (unpublished).

One study of patients receiving T cell-depleted marrow showed that antibody titers against tetanus toxoid or hepatitis B virus could be increased at least temporarily during the first 100 days by immunizing either the donor or the recipient before transplantation (357). The highest titers were found when both the donor and the recipient were immunized. It was suggested that activated natural killer cells present in the early posttransplant period were capable of secreting growth and differentiating factors for B cells and stimulating antibody production. Another study showed that antibodies against tetanus toxoid and diphtheria toxoid could be detected both during the first 100 days and more than 1 year after transplantation in patients receiving T cell-depleted marrow, even when neither the donor nor the host had been recently immunized before the procedure (358). These results suggested that long-term specific immunity could be transferred by T cell-depleted marrow.

There is evidence, however, suggesting an unusual impairment of immune function in some patients receiving T cell-depleted allogeneic marrow grafts. Fatal lymphoproliferative disorders often related to Epstein–Barr virus infection have developed in at least 11 patients after transplantation of T cell-depleted marrow (359,360). This complication occurs rarely in patients receiving unmodified marrow, but in this setting, associated risk factors have been HLA incompatibility, severe prolonged GVHD, and protracted immunosuppressive therapy (232,361,362). In Seattle, 2 of 52 patients transplanted with HLA-identical T cell-depleted marrow developed fatal EBV-associated lymphoproliferative disorders. In both cases the syndrome developed at 5 months after transplantation, and in both cases the patients had received three doses of posttransplant methotrexate. The first patient developed grade II GVHD and was treated with cyclosporine, whereas the second patient had no GVHD but did have persistent host-type lymphoid and hematopoietic cells. The first patient died of pulmonary failure caused by infiltrating immunoblasts of donor origin. The second patient, in whom the immunoblasts were of host origin, died from complications of treatment.

Specific risk factors predisposing for this complication in patients receiving T cell-depleted marrow have not yet been identified. Some investigators have suggested that prophylactic treatment with acyclovir might prevent this complication. The two patients described above, however, both received acyclovir for prophylaxis or treatment of herpes simplex infection. The first patient received 500 mg/m^2 (adjusted for renal impairment) for the first 30 days after transplantation, and the second patient received similar doses until day 21. The second patient also received acyclovir for 3 weeks between days 88 and 120 after transplantation for treatment of varicella zoster.

V. Graft versus Leukemia

The possibility that a marrow graft might contribute an anti-tumor effect (i.e., graft-versus-leukemia or GVL) was one of the considerations that prompted investigations of marrow transplantation for treatment of hematologic malignancies (363). A number of mechanisms could theoretically account for GVL (see Ref. 364 for review). (1) Alloantigens (minor histocompatibility antigens in HLA-identical grafts, MHC antigens in HLA-incompatible grafts) expressed by the malignant cells might serve as direct targets for GVH effector cells. (2) Leukemic cells might be eliminated through an "innocent bystander" effect of GVHD. (3) Leukemic cells might express unique antigens

which can serve as targets for immunocompetent donor cells. (4) Certain products of a GVH reaction such as lymphokines might have growth or differentiation effects on leukemic cells.

Some evidence for a GVL effect in humans has come from anecdotal reports of striking tumor regression during episodes of severe acute GVHD (365). Occasionally, Ph-positive metaphases have been transiently detected after transplantation for chronic myelogenous leukemia (22). Although data are preliminary, the observation that not all such patients have the subsequent development of overt leukemia suggests an active control mechanism.

Most of the data for a GVL effect have come from retrospective observations that leukemic relapse occurs less frequently in patients who develop GVHD (366–370). Weiden *et al.* (366) found the effect to be most pronounced in patients with lymphoid malignancies and in patients with a high leukemic burden (i.e., in relapse) at the time of transplantation. Allogeneic transplant recipients who did not develop GVHD and syngeneic (identical twin) recipients had similar relapse rates. The difference in relapse rates between patients with and without GVHD was most evident during the first 130 days after transplantation. An effect was not evident during the period between 130 and 260 days after transplantation, but again became evident after 260 days. A subsequent analysis of patients who survived the first 150 days showed that the effects of acute and chronic GVHD on the relapse rates were equivalent and additive (367).

With the advent of better supportive care and specific therapy for acute and chronic GVHD, it became possible to translate the antileukemic effect associated with GVHD into improved survival (368). Between 1970 and 1976, patients with advanced leukemia who had moderate to severe acute GVHD had worse survival that patients with minimal or absent GVHD. For patients with advanced leukemia transplanted during 1977 and 1978, the development of moderate to severe acute GVHD was associated with improved survival. The best survival was seen in patients who had *de novo* onset of chronic GVHD (367).

These retrospective observations formed the basis for a prospective randomized trial designed to determine whether the risk of leukemic relapse could be decreased by measures that would likely increase the incidence of acute GVHD (240). Patients with advanced hematologic malignancy of all types were randomized in three groups to receive methotrexate on days 1, 3, 6, and 11 only, or to receive methotrexate according to the conventional schedule with or without irradiated donor buffy coat infusions. Patients receiving methotrexate ac-

cording to the conventional schedule had a 25% cumulative incidence of acute GVHD, those receiving the attenuated schedule had a 61% incidence of acute GVHD, and those receiving buffy coat had a 90% incidence of acute GVHD. The incidence of chronic GVHD showed a parallel increase with acute GVHD. The unexpected and still preliminary result was that the projected relapse rates at 3 years after transplantation were similar in the three groups (34–44%), and relapse-free survivals were identical (27–38%). In this trial, it appears that the deliberate induction of GVHD or increased virulence of GVHD per se did not provide an increased anti-leukemic effect.

The above clinical results are consistent with experimental data suggesting that GVHD and GVL might represent independent effects of genetic disparity. GVL and GVHD do not always correlate in transplantation between various strains of mice (371). Furthermore, a GVL effect can be observed in mice given T cell-depleted allogeneic marrow and in mice transplanted under gnotobiotic conditions where GVHD is reduced (372–375). In one carefully studied marrow transplant recipient, it was possible to isolate clones of donor-derived lymphoid cells that showed spontaneous cytotoxic activity against cryopreserved host leukemic cells (376). The clones also had cytotoxic activity against NK-sensitive cell lines but had no activity against activated T lymphocytes from the host. It was suggested that cells of this type might be capable of having an anti-leukemic effect without causing GVHD. Thus, measures taken to prevent GVHD might not have an adverse effect on the risk of leukemic relapse. A preliminary suggestion that this might be the case has come from the observation that the decreased incidence of acute GVHD associated with the use of methotrexate plus cyclosporine has not yet been accompanied by an increased incidence of relapse (248).

Results of two published studies have suggested an increased relapse rate in patients receiving T cell-depleted HLA-identical marrow. In a retrospective study, Apperley *et al.* (377) found an increased relapse rate in patients transplanted with T cell-depleted marrow during the first chronic phase of CML compared to similar historical control patients transplanted with unmodified marrow. Mitsuyasu *et al.* (127) found a similar trend in a prospective randomized study of patients with ANL or ALL in remission or CML in various phases. These data suggest that certain T cells in the donor marrow may be capable of eliminating residual leukemic cells in the host. Further clinical studies will be needed in order to confirm or refute these preliminary findings and to determine which types of leukemia show susceptibility to control by engrafted T cells. As yet, there is no evi-

dence from human studies to indicate whether GVHD and the GVL effect are mediated by a single population or by separate populations of T cells.

VI. Conclusions

The past four decades have been marked by enormous progress in developing marrow transplantation from its origins as an experimental animal model to its present application as the therapy of choice for certain human diseases. Despite this progress, there is clearly room for improvement, since leukemic relapse, infections with or without GVHD, difficulty with engraftment, and toxicity caused by treatment regimens still remain frequently encountered problems. To date, the single most effective measure for reducing the incidence of recurrent leukemia has been to initiate marrow transplantation earlier in the course of the disease. Leukemic relapse remains the single most frequent cause of failure in patients transplanted for advanced stage hematologic malignancies. Thus, identification of more effective, yet tolerable pretransplant conditioning regimens must be the first priority for the near future. Trials are under way testing new drugs for treatment of infections, particularly those caused by viruses. In parallel with these efforts, the possibilities of enhancing immunity by active immunization of the donor or recipient or by passive adoptive therapy are being explored. Improved posttransplant immunosuppressive regimens for prevention of acute GVHD have been developed recently, and further refinements may be possible.

The prospects and future role for T cell depletion remain to be defined. The major issue currently rests on whether sustained engraftment and GVHD represent the effects of a single T cell population or whether distinct populations are involved. Experiments addressing this question are urgently needed in animal models where the genetic disparity can be precisely controlled, where reagents for separation of T cell subsets are available, and where long-term chimerism of both lymphoid and hematopoietic cells can be assessed serially. If graft enhancement and GVHD represent the effects of a single population of T cells, then clearly the only practical approach for using T cell depletion in humans would be to develop more immunosuppressive, yet tolerable, pretransplant conditioning regimens. This effort might be especially rewarding if greater immunosuppression were accompanied by improved elimination of leukemic cells. Omission of posttransplant immunosuppression might reduce toxicity associated with more vigorous pretransplant conditioning.

However, if graft enhancement and GVHD represent effects of separate cell populations, it may be possible to achieve selective T cell depletion which prevents GVHD without the increased graft failure seen with current pretransplant conditioning regimens. Striking structural homologies have been found between the T cell surface antigens of different species, and cells expressing homologous antigens appear to have analogous functions. Thus, identification of markers capable of distinguishing cells that cause GVHD from cells that facilitate engraftment in an appropriate animal model might quickly lead to successful clinical trials in humans. Once methods for assuring reliable, durable engraftment of T cell-depleted marrow have been identified, randomized prospective clinical trials can be designed to assess the effects of T cell depletion not only in terms of acute and chronic GVHD but also in terms of immune reconstitution and infectious complications, leukemic relapse, and ultimately, survival.

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