REDUCED IN VITRO IgG SECRETION FOLLOWING IN VIVO INJECTION OF INTERFERON (WELLFERON ^{R}) IN MULTIPLE SCLEROSIS PATIENTS

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Abstract

An in vitro IgG secretion assay was developed to investigate the regulation of the humoral immune response in humans. Pokeweed mitogen (PWM), a plant lectin derived from Phytolacca americana stimulates human peripheral blood mononuclear cells (PBMNC) to divide and resting B-lymphocytes to differentiate into immunoglobulin secreting cells (ISC). This differentiation requires that both monocytes and T-lymphocytes be present in the culture system. The amount of IgG secreted by these differentiated B-lymphocytes in response to PWM appears to be the net result of a balance between the functional activity of the regulatory T-helper and T-suppressor cells. Alterations, qualitative or quantitative in any of these leukocyte subsets could conceivably alter the amount of IgG secreted by the B-lymphocyte subpopulation.

We have employed this assay to investigate the immune status in a group of chronic progressive multiple sclerosis (MS) patients and to assess the immunoregulatory effects of interferon (Wellferon R, INF) administered in vivo to this selected group. Their mononuclear cells (MNC) were studied in this PWM induced IgG secretion assay before INF treatment and again after 7 days of daily sub-cutaneous injections (5 X 10^6 u/day). Twenty patients received the interferon (INF) preparation and eighteen received normal saline. The study was carried out in a double blind manner and the code was broken only after individual results had been calculated.

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The level of IgG secreted by PBMNC in response to PWM was measured in: 1) patients before treatment; 2) healthy volunteers; and 3) placebo and INF treated patients. We found that the level of IgG secreted into the supernatants after 7 days was significantly higher in the MS group than in the control group. We attribute this to an alteration of the normal immune response in MS patients. Following one week of daily subcutaneous injections, the level of IgG secreted by the MNC isolated from INF treated patients was dramatically reduced compared to; 1) the level observed prior to treatment and 2) the amount of IgG secreted by the MNC isolated from the placebo treated patients. This is the first report on the ability of INF administered in vivo to modulate the in vitro humoral immune response in humans.

Using techniques to separate and study the function of lymphocyte subsets we have searched for the subset affected by in vivo INF injection and found that the INF induced decrease in IgG secretion seems to be mediated by an effect on B cells.

These studies centered on IgG secretion. In order to investigate suppressor cell function we measured the suppression induced by in vitro Con A stimulated lymphocytes on the proliferative response of mitogen stimulated heterologous lymphocytes. Confirming previous reports of decreased suppressor activity in MS patients we found the level of suppression significantly lower in the patients studied vs the controls. However this Con A induced suppressor activity was not affected by in vivo injection of INF.

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Introduction

Immunology and Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). The clinical course of the disease is extraordinarily variable but generally presents itself as a series of relapses and remissions or as a chronic progressive disease. The pathological reactions responsible for the observed symptomatology is peri-axial demyelination affecting the central nervous system. The mechanism responsible for the observed destruction of myelin and oligodendrocytes however remains a mystery.

Characteristic lesions of an MS brain are well demarcated focal zones of demyelination or "plaques" (32). Plaques are distributed throughout the white matter of the brain and spinal cord with a predilection for certain areas within the CNS, especially the periventricular area. (For reviews see (2,134)). The plaques may vary in age, size and shape and are quite often located perivascularly. Despite many publications referring to the "early lesion" in MS, very little is known of the initial stage of plaque evolution. The lack of knowledge in this area stems from the chronic nature of the disease. This has forced pathologists to investigate early lesions by studying either the edge of a plaque or the unaffected white matter. It could be noted here that within the macroscopically normal appearing white matter, Allen et al. (2) observed that 72% of the histological sections which they studied were abnormal, the most frequently observed abnormality being diffuse gliosis.

Older plaques are grey due to loss of myelin and have an altered consistency as a result of gliosis (134). Within the plaque, demyelinated axons, numerous astrocytic processes and very few oligodendroglia are observed.

Unlike established plaques, early lesions do not have well demarcated edges. This is due to oedema and the presence of inflammatory cell infiltrates at the site of demyelination (134). The cell infiltrates which often surround small blood vessels consist of lymphocytes, macrophage, and plasma cells. The distribution of these cells within the plaques and normal appearing white matter has recently been investigated.

Hauser et al., (67) observed that Ia bearing cells (activated T-cells, B cells and monocytes) are present throughout the normal appearing white matter. Using monoclonal Abs directed against specific lymphocyte subsets, Traugott (185) observed large numbers of OKT4+ cells (T-helper lymphocytes) at the lesion margins and extending well into the adjacent white matter. The OKT8+ cells (T-suppressor/cytotoxic lymphocytes) were preferentially distributed around small blood vessels at the lesion margins. Within the centre of the lesions Ia+ macrophage were most numerous. Prineas et al. (135) also observed plasma cells in the unaffected white matter and around the perivascular compartment of typical chronic lesions. The proximity of T4+ cells to Ia+ macrophage and plasma cells in the absence of the OKT8+ T-suppressor cells within the normal appearing white matter suggests that there may be a lack of control in down-regulating immune function in the CNS of MS patients.

The etiology of MS remains an enigma. One hypothesis suggests that MS is an autoimmune disease directed against an as yet unidentified antigen present on myelin or oligodendrocytes. Another hypothesis suggests MS is due to a chronic viral infection within the CNS. The two hypotheses are not mutually exclusive and one could hypothesize that an abnormal immune response to a chronic viral infection in genetically susceptible individuals results in the autoimmune destruction of myelin.

There is a plethora of data documenting abnormal immune phenomena in the serum and cerebral spinal fluid (CSF) of MS patients (for reviews see refs, 78,124). There are two such abnormalities currently recognized as diagnostically important in MS: 1) intrathecal IgG synthesis or secretion, and 2) the presence of oligoclonal bands of IgG detected in the CSF and occasionally in the serum, when such samples are subjected to electrophoresis.

Increased intrathecal IgG secretion is present in 74% (124) to 92% (78,180) of patients with clinically definite MS. Elevated levels of IgG in the CSF can in addition to intrathecal IgG synthesis, be the result of a damaged blood brain barrier (bbb) or high serum IgG (78). The latter two factors would result in the diffusion of IgG from the blood to the CSF since the concentration of IgG is higher in the blood. The concentration of IgG in MS serum however is usually normal (98) and the blood brain barrier is only affected in approximately 20% of MS patients (179).

As albumin is synthesized in the liver only, a comparison between the amount of IgG and the amount of albumin in the CSF allows one to differentiate between intrathecal IgG production and bbb damage. Normal

albumin levels in the CSF in the face of elevated IgG levels in the CSF is indicative of intrathecal IgG secretion. There have been many indices proposed to measure intrathecally secreted IgG (124) but the most popular index for diagnostic purposes is that proposed by Tourtellotte (181). This index is a measure of the amount of IgG secreted intrathecally per day and takes into account both the effects of high serum IgG and a defective bbb. The values obtained by this method demonstrate abnormally high levels in approximately 65% of all MS patients and 92% of clinically definite MS patients (180).

When the CSF or brain eluates of MS patients are subject to electrophoresis discrete bands of IgG (oligoclonal bands) appear. About 90% of MS patients' CSF reveal these discrete oligoclonal bands of IgG in the gamma area of CSF electrophoresis (105). Although these bands are of diagnostic importance, this phenomena is not specific for MS (for review, see ref. 112). Oligoclonal banding has been demonstrated in a variety of neurological inflammatory conditions associated with particularly intense or chronic antigenic stimulus (112). One theory is that these oligoclonal bands of IgG present in the CSF, brain and occasionally (27%) in the serum (105) of MS patients represent the products of a few clones of activated B-lymphocytes, and that the immunoglobulins which make up these bands are associated with the etiological agent (90,96). Support for the latter concept is suggested by data obtained in the study of subacute sclerosing panencephalitis where the oligoclonal IgG is specific for the etiological agent, namely measles virus (97,190,191).

In the CSF of MS patients measles virus Ab are found more often than in controls. This observation was first noted by Adams et al. (1)

in 1962 and has been amply confirmed (42,158,159). Antibodies to other viruses are also found more often in the CSF of MS patients than in other neurological controls (120,158). The presence or absence of these Ab does not correlate with disease activity (42,120). Additionally, antibodies to more than one virus have been found simultaneously increased (12,158). These latter observations coupled with the identification of anti-oligodendrocyte Ab and elevated IgG secretion in the CSF suggests that this immune response is non specific and that possibly an abnormality exists in the regulation of B-cell function. That a specific Ag does exist and is the target in the demyelination process cannot be ruled out however since it has been reported that a specific immune response (shown as an increase of a specific antibody) can be accompanied by many non-specific immune responses (ie, increased levels of non-relevant antibodies) (124).

It has also been suggested that the oligoclonal bands represent a secondary phenomenon occurring as a result of the destruction of myelin in the CNS (78). Obviously the pathogenesis of the bands is obscure and it remains to be resolved whether a specific Ag is involved or if the oligoclonal bands that are observed in the CNS of MS patients are the result of a deregulation in normal immunological control mechanisms.

Of particular interest to our group is the demonstration of abnormal immune regulation in the peripheral blood of MS patients. The immune system can be grossly divided into two main components, effector cells and regulatory cells. The former include B-cells which secrete immunoglobulin, cytotoxic T-cells which mediate cytotoxicity against allogeneic targets, K-cells (K=Killer) responsible for antibody mediated

cellular cytotoxicity and NK (Natural Killer) cells which mediate cytotoxicity against tumor cells and virus infected cells. The level of activity of these effector cells appears to be the result of the functional state of the regulatory T-cells. The regulatory cells are composed in part of helper or inducer T-cells and suppressor T-cells, the functions of which are self explanatory. These cells bear different surface markers roughly associated with their function and which permit their enumeration. Commercially available monoclonal Ab's directed against these cell subset specific surface markers (See Table 1) have recently enabled investigators to isolate these subsets and analyse their respective functions in vitro.

There are numerous studies demonstrating altered numbers (18,28,76,136,146,160) and function (5,7,13,61,118,198) of suppressor/cytotoxic cells in the peripheral blood of MS patients. Before the advent of monoclonal Abs, B and T cells were quantitated and isolated by the presence of surface Ig and by binding to sheep red blood cells (SRBC, i.e. E-rosetting) respectively. The number of surface Ig positive cells (B-cells) appears to be normal in the blood of MS patients, however the number of E-rosetting (T-cells) cells is reduced (99,122,186). Oger et al. (122) and Utermohlen et al. (189), observed that the avidly rosetting T-cells (a subset of all rosetting T-cells) were reduced in active MS. The T-suppressor/cytotoxic lymphocyte subset bear more receptors for SRBC than does the T-inducer subset, suggesting that this suppressor T-cell subset is selectively altered.

The regulatory T-helper and T-suppressor subsets can also be roughly divided into two groups based on the presence of cell surface receptors for the Fc fragment of either IgG (TG-cells) which mediate

suppression or IgM (TM-cells) which represent the inducer cell subset (114). The TG subset reportedly fluctuates with disease activity (76,77,186).

Using the currently available monoclonal Ab's (see Table 1) recent studies have demonstrated a reduction in the level of circulating Ts-cells (OKT8, OKT5, Leu 2),(10,18,28,68,130,146) in MS patients measured either as a percentage of all peripheral blood lymphocytes or as a ratio of T-helper to T-suppressor cells (i.e. OKT4:OKT8). This observation has been confirmed in our laboratory (131) however the extent of the reduction in the number cells bearing the suppressor phenotypes remains to be determined. Changes in suppressor cell numbers vary with different forms of the disease (131,136). It appears that approximately 40% of the chronic progressive patients and 70% of patients in acute relapse have a decreased number of OKT8+ cells whereas stable MS patients have normal values (68,124,130,157).

The mechanism responsible for the observed decrease in the number of lymphocytes bearing suppressor subset specific surface markers is not known. That these cells are destroyed or preferentially lost to the CSF does not agree with the rapid variations in the proportion of T8+ cells observed, and the number of T8+ cells in the CNS hardly accounts for the decreased levels in the blood (68,124). A more likely explanation for the reduction of suppressor cell numbers is a modulation in the expression of the cell surface markers (8,131). An autoantibody binding to the surface of the suppressor cell subset could conceivably alter the binding of the Ts-cell specific monoclonal Abs. If this were the case, the suppressor cell would still be present however it would not be

recognized by conventional methods (8). Support for this mechanism stems from the occurence of anti-lymphocyte Ab which have been detected in the serum of some MS patients (89,100,107,164).

Studies of the percentages of OKT8+ cells and Leu 2a+ cells (separate markers both identifying suppressor/cytotoxic lymphocytes) suggest that the OKT8 marker fluctuates much more than the Leu 2a marker, (which does not appear to vary with clinical changes in the disease) (131). From these results it seems likely that the observed apparent reduction in suppressor cells in MS patients results from modulation of the surface Ag's (124).

Not only are the T suppressor cell markers reduced, suppressor cell function is also altered in MS. It appears that functional alterations parallel the clinical course of the disease (7,13,18,68,76,77,146). Con A induced suppressor cell activity has consistently been demonstrated to be reduced in patients with MS (7,13,61).

Several groups using different assays to measure suppression have noted aberrant regulation in MS (77,118,198). Low doses of irradiation abrogates Ts function (169). Increased IgG production in pokeweed mitogen stimulated cultures of irradiated T-cells plus B-cells above the level IgG produced by non-irradiated T-cells plus B-cells is indicative of active suppression. Huddlestone took advantage of this phenomena to demonstrate increased suppressor activity immediately following clinical relapse (77). Similarly other groups have observed increased PWM induced IgG secretion in the PBMNC cultures of MS patients vs healthy control individuals and speculated that this was due to either decreased suppressor cell activity or excessive helper activity (10,62,82,93,123).

As suppressor cells are thought to be involved in the maintenance of self tolerance, and as these cells have been shown to fluctuate with the clinical course of MS it is conceivable that once the initial pathological event occurs, be it viral or otherwise, the aberrant immune regulation observed could contribute to the relapsing and remitting nature of this disease.

Other immunological phenomenon reported to be altered during the active phase of the disease include: decreased NK cell activity (109) along with decreased responsiveness to INF (119), and increased cellular cytotoxicity by K cells as measured by ADCC (53,110).

It remains to be resolved whether the observed alterations in the immune systems of MS patients are the primary cause of the disease or the secondary result of a primary pathological reaction. Regardless of which mechanism proves to be responsible for the initiation of the disease, it is possible that the abberrant immune regulation observed is contributory to the self perpetuating nature of multiple sclerosis. Therefore future investigation should explore the notion of abnormal immune regulation in response to common viral or self Ag's as a mechanism responsible for the relapsing and remitting nature of this disease.

II. Pokeweed Mitogen Induced IgG Secretion In Vitro: A Model of the In Vivo Humoral Immune Response.

Immunoglobulin (Ig) production and secretion is actuated by the B-lymphocyte subset. B-lymphocytes can be defined as cells derived from lymphoid hematopoeitic precursors in the fetal liver and adult bone marrow (55) endowed with the ability to synthesize immunoglobulin (195).

B-cells are very heterogeneous, ranging from primitive precursors to fully differentiated plasma cells, with concomitant changes in the surface markers expressed (188), in patterns of recirculation and homing into secondary lymphoid organs (95,173), in their lifespan (111), in class of Ig produced (201), and in their ability to respond by proliferation and/or Ig secretion after interaction with the appropriate mitogen or antigen (Ag).

B-cells can be defined histologically according to their state of maturation and the following classification is generally accepted.

1) Precursor B-cells or pre B-cells: No detectable surface immunoglobulin however cytoplasmic IgM is detectable and these cells will differentiate into mature B-cells (65).
2) Resting B-cells: Small (7-9 ums) Ig-positive cells that are competent to synthesize Ig molecules some of which will be deposited on the cell membrane to serve as antigen-binding receptors (54,113).
3) Activated B-cells: Large blasts or plasma cells which histologically demonstrate well developed rough endoplasmic reticulum and golgi apparatus. These large cells develop after the activation of resting B-cells and are able to synthesize Ab

molecules at a high rate and export them extracellularly in large quantities (108).

Resting B-cells can be activated to divide and/or produce immunoglobulin. It is the mechanism by which the B-cells are triggered which differentiates *in vitro* pokeweed mitogen induced B-cell differentiation from Ag induced differentiation *in vitro* and *in vivo*. The Ag-specific binding receptor on the B-cell surface is identical to the Ab molecule that the cell is capable of secreting after activation. Selective binding of Ag to cell(s) bearing receptors is the key to specific activation of B-cells. Once the specific binding has occurred the cell(s) (only a very small fraction of immunocompetant B-cells) becomes activated to expand clonally and to differentiate into a state where it can secrete large amounts of specific Ab. Mitogen activation of lymphocytes on the other hand differs from the above Ag specific activation in that the number of B-cells activated is several orders of magnitude higher than the number of precursor cells reported for each Ag-specific clone (37,113) and the multiplicity of antibody specificities found after stimulation is also much larger. The large variation in the specificity of the Ab secreted strongly suggests that PWM triggers B-cell differentiation independently of the Ig specific receptor site. Nothing is known of the physicochemical structure of the mitogen receptor.

Although these differences in the triggering of resting B-cells exists, it appears that once the initial event occurs, the mechanism responsible for proliferation and differentiation is the same in both systems. Once activated, mitogen stimulated immunocompetant cells perform equally well all the functions that antigen-activated cells

perform (3,63,64). For the above reasons mitogen induced differentiation of resting B-cells into immunoglobulin secreting cells (ISC) has been used extensively to unravel the complex mechanism of lymphocyte activation.

Once stimulated, resting B-cells generally divide and differentiate into ISC. Cell division however is not always accompanied by immunoglobulin secretion (151) and conversely extensive Ig secretion is not necessarily associated with cell division (115,162). From these observations one could postulate that proliferation and differentiation of resting B-cells into ISC after stimulation are two separate phenomena (49,71).

When measuring B-cell activation therefore one must be aware that different methods may measure different cell functions.

B-cell activation is currently measured as:

a) a proliferative response, measured by the uptake of radiolabelled DNA precursors (e.g. ^{3}H Thymidine).

b) an enumeration of Ig secreting cells, measured by either intracytoplasmic staining (36,54) or the reverse hemolytic plaque assay (84).

c) a quantitative assessment of the immunoglobulin secreted into the supernatant, measured either by ELISA (195b) or radioimmunoassay (197).

The above assays are usually used to measure non-specific B-lymphocyte activation. Assays have been developed which do measure Ag-specific Ab responses (Rev. in 55).

The remainder of this review will concentrate on the activation and regulation of B-cell differentation in PWM stimulated human peripheral blood mononuclear cells (PBMNC).

Pokeweed mitogen, a plant lectin isolated from Phytolacca americana, stimulates T-cells to divide (63) and induces the polyclonal activation of resting B-cells manifested by their proliferation and differentiation into ISC (204). PWM binds to the B-cell surface although this is by itself insufficient to induce differentiation (54). T-cells (47,80,83) as well as macrophages (43,153,154,155) are required for the PWM induced differentiation of resting B-cells into ISC. It was originally argued that macrophages were not required for B-cell differentiation in this system (47,79,162) however it is now generally accepted that they are required for the generation of ISC. The most probable cause for the conflicting results stems from the efficacy of macrophage depletion (22). Contamination by only 1-2% macrophages in high density cultures is sufficient to restore the polyclonal B-cell response to PWM (153). Most notably it appears that macrophages have two distinct functions in this system. The first requires only the presence of intact cells and the second involves active secretion of lymphokines, most likely IL-1 (155). Both these functions are required for the complete differentiation of resting B-cells into ISC (155). Only intact macrophages however are required for the PWM induced proliferative response of the PBMNC.

Interestingly, the accessory cell role of the macrophages and their secretory products is not genetically restricted in this system as allogeneic macrophages are able to fully reconstitute both the PWM induced generation of ISC (153) and the mitotic responsiveness (43).

In macrophage reconstitution experiments it is interesting that the optimal response exceeds the response observed in the normal cell cultures, however once a critical percentage of macrophages has been exceeded the level of response observed is significantly reduced (153-155).

Macrophage and/or their secretory products cannot however reconstitute the PWM induced generation of ISC if T-lymphocytes are not present in the culture system (47,79,83). Using the plaque forming cell (PFC) response as a measure of B-cell differentiation it was observed that depletion of the T-cell subpopulation in both peripheral blood and tonsillar mononuclear cell cultures markedly reduced B-cell differentiation following PWM stimulation. The response was fully reconstituted by adding back T-cells (47,83). T-cell supernatants on the other hand could reconstitute the response of the T-cell depleted tonsillar lymphocyte cultures but not that of the cells from the peripheral blood suggesting a more complex T-cell requirement in the PWM induced response in the PBMNC cultures (47).

The level of the response induced by PWM and measured either as the number of ISC or the amount of Ig secreted, varies markedly from one individual to the next. The variation in the responsiveness of the cells from any given individual when assayed over a fairly long period of time however varies much less by comparison (83). Some individuals are consistently "high responders" while others are consistently "low or non-responders". It appears that the level of responsiveness in this system is not due to an incapacity of the B-cell subpopulation to differentiate but is a result of the balance between the helper and suppressor influences in the regulatory T-cell subsets (9,70,124). In

man these regulatory T-cell subsets have been recognized for a number of years. They have been identified by hetero-and auto-immune antisera (142), differential Fc receptor binding (114), and most recently by the production of monoclonal Abs (137) which recognize subset specific surface markers. The recent production of these monoclonal Ab's specific for T-cell subsets at functionally different stages of maturation provides powerful tools for the identification and isolation of these regulatory T-cells subsets (See Table 1)

The T-cell subsets defined by the surface markers OKT4, T_M (Fc receptor for IgM) and Leu 3a contain the helper/inducer activity necessary for the PWM induced differentiation of B-cells (114,138). T-cell subsets identified by the surface markers OKT5, OKT8, T_G (Fc receptor for IgG) and Leu 2a (15-30% of peripheral blood T-cells) suppress the generation of plasma cells when added together with T-helper cells, B-cells and PWM.

Isolation of these subsets using mAbs (81,137,156,174,205) and other less conventional means (47,70,83,114,193,199) have shown that in addition to T-B cell interactions, precise interactions among functionally distinct T-cell subsets ultimately determine the net outcome of the T-cell immunoregulatory influence on B-cells.

The role of the suppressor T-cell subset in terms of influencing the level of response observed after PWM stimulation was investigated in separate laboratories using different methods to isolate the suppressor T-cells. One involved isolating the T_S-cell subset from high responders and non-responders by virtue of the presence of the surface receptor for the Fc fragment of IgG (T_G). Addition of the T_G cell

subsets in allogeneic co-cultures demonstrated that the T_G cells (T_S) from non-responders suppressed the PWM response to a much greater extent than the T_G cells isolated from high responders (69). In another assay the T-cell subsets were purified by virtue of the mutally exclusive cell surface markers OKT4 and OKT8 (T_H and T_S cells respectively) (156). This group observed a significant correlation between the amount of IgG produced in whole MNC cultures and the level of suppression induced by the T8 cells isolated from the same individual; i.e., the addition of OKT8+ cells isolated from non-responders to a pool of high responder B-cells, monocytes and T helper cells suppressed PWM induced IgG secretion to a much greater extent than did the addition of OKT8+ cells isolated from high responding individuals. Further support for the role of OKT8+ cells in actively regulating the level of response induced by PWM is derived from studies which demonstrate that the removal of this subset results in significantly enhanced responses (81).

It would appear from these results that the level of response is dependent on the amount of suppressor immunoregulatory cell function that is present or has been spontaneously induced as opposed to the absolute number of cells in the T-suppressor compartment (10,156,193).

It is becoming increasingly evident that the currently recognized T-cell subsets are composed of more than one homogeneous population (59,147,175,193,205). Therefore although the absolute number of cells within the subsets recognized by MAbs may not be responsible for the variations observed in the level of IgG secreted in response to PWM one must consider the possibility that sub-subsets within the T_S cell subset may not be equally represented in responder vs non-responder individuals.

The mechanism of suppression actuated by the regulatory T-cells is likely to be very complex. It is well documented that irradiation (114,169) as well as other methods of preventing in vitro blastogenic responses (49) inhibit the activity of suppressor cells and result in increased levels of responsiveness to PWM in PBMNC cultures. It appears that while T_H cells are resistant to fairly high levels of irradiation (5,000 R) (48), T_S cells are sensitive at lower doses (1,500 - 2,000 R) (48). One can conclude from these results that T-helper cells can function in the absence of blastogenesis while T_S-cell activity is closely linked with cell division (49).

In PWM stimulated cultures of PBMNC it would appear that the OKT8+ T_S cells regulate IgG production by acting at the level of the T_H cell. In a complex investigation of the T-T and T-B interactions involved in the PWM induced B-cell differentiation to ISC, Thomas et al (174-177) observed the following: (1) T-helper functions are contained exclusively within the OKT4+ subset and contrary to previous reports this subset is radiosensitive except at high T:B ratios (i.e. > 1); and (2) The OKT8+ subset is devoid of helper function but expresses a suppressor function that requires the presence of the radiosensitive OKT4+ subset to function. Interestingly, investigation of patients with T-cell leukemias whose blasts suppress B-cell differentiation revealed that these leukemic cells also require the presence of radiosensitive normal T-cells to induce the suppression of B-cell differentiation (29b). These results indicate that in PWM stimulated cultures of PBMNC, OKT8+ cells act at the level of the T_H cell subpopulation to inhibit the differentiation of B-lymphocytes. At the present time the precise mechanism by which the

OKT8+ cells suppress OKT4+ dependent B-cell differentiation is not known however the following mechanisms can be readily envisioned. OKT8+ T-cells may interact directy with preformed helper factors or compete with helper factors for their effect(s) on B-cells (177). This possibility is supported by the results of Palacios et al. (125) who showed that concanavalin A (Con A) activated suppressor T-cells could absorb out the T-cell growth factor IL-2. Furthermore Jones' group demonstrated that the OKT8+ population regulated the growth of B-cells, T_H cells, T_S cells and monocytes (81). It seems possible therefore that after PWM stimulation OKT8+ cells might regulate the level of response in PBMNC cultures by the absorption of growth factors from different cell types. Thomas et al (177) however demonstrated that OKT8+ cells did not suppress B-cell differentiation in the presence of preformed helper factors. As these helper factors were generated from allogeneic culture one must consider that perhaps PWM may be more potent than helper factor in activating the OKT8+ suppressor/effector function.

Other mechanisms by which the OKT8+ population may interact with other subpopulations include the production of suppressor factors that act directly on the T_H cells or alternatively act on non-T-cells (i.e. monocytes) that may play a role in OKT4+ dependent helper factor production.

Obviously further studies of distinct immunoregulatory cell subsets are required in order to elucidate the precise cellular interactions involved in the regulation of PWM induced IgG secretion in humans.

We can conclude that in the PWM induced differentiation of resting B-cells into ISC both intact monocytes, T-cells and their secretory

products are required. The level of response appears to be dependent on the functional state of the radiosensitive suppressor T-lymphocyte subpopulation.

Exploring the final mechanism of regulation in this system should be rewarding because as we have seen earlier aberrant IgG secretion within the CNS as well as abnormal suppressor cell functions in the peripheral blood are the main biological abnormalities recognized in MS patients to date.

III. Immunomodulatory Effects of Interferon

In 1980 an international group of scientists met at the National Institute of Health in Bethesda, Maryland to define what was meant by "interferon", to classify the various interferons that had been discovered, and to devise a system for their orderly nomenclature. The committee decided on the following definition for interferon: "To qualify as an interferon a factor must be a protein which exerts virus non-specific, antiviral activity at least in homologous cells through cellular metabolic processes involving synthesis of both RNA and protein" (170).

The interferons were classified according to their antigenic specificities and the type designations are now alpha (α), beta (β) and gamma (γ) corresponding to previous designations of leukocyte, fibroblast and type II (immune) interferons respectively.

In addition to the classical anti-viral and anti-tumor activities, interferons (IFN) possess immunomodulatory properties (for review see ref. 41).

We will focus on the immune modulating effects of those INF produced and secreted by the Nawalma lymphoblastoid cell line, namely alpha IFN and a small amount of beta-IFN. Conventionally the interferons produced by lymphoblastoid cells have been regarded as forming a separate category. However just as the IFN genes present in lymphoblastoid cells are the same as in circulating B-lymphocytes, their products should be the INF of the appropriate types namely alpha and beta (51).

Cloning of the alpha IFN genes in bacteria by recombinant DNA techniques have revealed 9 distinct leukocyte (alpha) INF subtypes that

are coded for by at least 10 genes (58). Studies using monoclonal Abs suggest that two forms of the beta-INF exist (74). In light of the remarkable heterogeneity within the INF produced, one must consider that each subtype could potentially exert a specific biological effect, or alternatively that synergistic effects could occur between different subtypes. Most of the earlier studies on the immunomodulatory effects of INF used only crude preparations of partially purified INF's, which made it difficult to separate the immunomodulatory effects of INF from those of other biologically active substances (eg. lymphokines) contaminating the preparations. Considering these two points one must be very cautious when interpreting the results of earlier studies of the immunomodulatory effects of INF.

Murine Experiments in vitro

Most investigations of immune modulating effects of INF involve the addition of INF to in vitro assay systems. In human and murine systems both alpha and beta INF have been shown to: enhance NK cell activity (91) and the generation of cytotoxic T-lymphocytes (72,207), inhibit DNA synthesis in lymphocytes stimulated with mitogens or antigens (166) and to modify immunoglobulin synthesis (24,29,29b,32,33,57,66,94,128,152b, 166,168,194) as well as protein synthesis (87).

The modulating effects of interferon on B-cell differentiation in murine systems are well documented and will be reviewed in the following section.

Gisler et al. (57) were the first to report that INF could affect B-cell function. Concentrations of murine L-cell INF induced by Newcastle Disease Virus (NDV) greater than 3,000 units per ml added to

mouse spleen cell culture resulted in a reduced plaque forming cell (pfc) response against sheep red blood cells (SRBC) as compared to cultures with control or mock interferon preparations. When lower doses of the INF preparation were added to spleen cell cultures obtained from low responding mice, the pfc response against SRBC was significantly increased.

Pretreatment of separated B-lymphocytes prior to Ag exposure resulted in a decrease in the number of pfc observed thus suggesting that this subset was directly affected by the INF preparation.

In a similar study Booth et al. (24) noted that relatively high doses of L-cell interferon induced by reovirus type III added to mouse spleen cell cultures reduced the number of pfc detected against SRBC compared to control cultures not exposed to INF. This groups also demonstrated that the presence of low doses of this INF preparation enhanced the pfc response against SRBC in cultures obtained from low responding mice.

The in vitro immune modulating effects of these INF preparations appeared to be species specific as rabbit interferon had no effect on the mouse spleen cell cultures.

B) Murine Experiments in vivo

The timing between INF injection and immune stimulation is critical in determining the effects observed. Mouse L-cell interferon induced by NDV (1.5×10^5 units (29b) and 1.8×10^5 units (33) divided into 4 doses and injected over 4 hours) was injected into mice 4-48 hours prior to Ag stimulation (SRBC). Similar results were recorded by both groups;

i.e. a marked reduction in the number of Ab producing spleen cells observed. This effect was again species specific and human interferon had no effect.

If however the interferon preparation was injected 48-96 hours following Ag injection enhancement of the Ab response was observed (29b). This latter group (29b) also investigated the effect of the in vivo interferon injection on the in vitro Ab response of spleen cell cultures stimulated with a T-cell independent mitogen derived from S. tiphimurium (lipopolysaccharide (LPS)). They noted a significant reduction in the PFC response to LPS in splenic cultures obtained from mice treated with interferon, again suggesting that the B-lymphocyte subpopulation is directly affected by interferon.

Virelizier etal. (194) injected animals with Sendai virus as a interferon inducer (peak circulating alpha interferon levels of 18,000 units/ml of sera 12 hours after inoculation) and noted a 97% reduction in the number of Ab forming spleen cells when the Ag (SRBC) was injected 12 hours after the virus inoculation. Interferon containing serum inhibited the in vitro plaque formation against SRBC in spleen cell cultures in a dose dependent fashion.

C) Human Experiments in vitro

Here also effects of human interferon preparations on the antibody response of peripheral blood mononuclear cells are dependent on the Ag, and the type, dosage and timing of the INF used.

Two groups using Cantell type interferon obtained from the Finnish Red Cross investigated its effects on the Ab response of human peripheral

blood mononuclear cells (PBMNC)(66,128). Both groups noted a time dependent effect on the Ab response. Parker (128) observed that if INF (100 and 500 units/ml) was added to PBMNC cultures 24 hours prior to Ag (HRBC), then washed away, a significant increase in the number of Ab producing cells was observed. If INF was present throughout the culture period (10-500 units/ml) the number of Ab forming cell detected was reduced. In a similar study Harfast (66) noted that if cells were pretreated with INF (100 units/ml up to 5,000 units/ml) the amount of IgG secreted in response to PWM was frequently increased, often by more than 100% over that obtained by untreated PBMNC. In contrast if INF was present throughout the 7 day culture period reduced production of IgG was observed.

Rodriguez et al. (152b) using purified human alpha interferon (10-100 units/ml)(Sigma Chemical Co., St. Louis, Mo.) added together with PWM to human PBMNC noted that the level of Ig (IgG and IgM) produced in culture increased. Preincubation of the PBMNC for 24 hours with the human alpha INF preparations (10-100 units/ml) also resulted in an increased level of Ig secreted.

The use of recombinant interferon is expected to clarify the issue. Shalaby et al. (166) recently reported that three different alpha INF subtypes when present throughout the culture period (100-1,000 units/ml) enhanced the Ab response of human PBMNC to SRBC.

Yen et al. (206) compared the effects of two different preparations of partially purified human alpha-INF (Cantell type) and 2 different species of human recombinant alpha interferon (INF alpha A and INF alpha D) on the level of Ab secreted by PBMNC in response to PWM. One of the

partially purified alpha INF preparations decreased IgG production at all concentrations tested (10-10,000 units/ml) when present throughout the 7 day culture period. The other preparation increased IgG production at concentrations of 10 to 30 units/ml. Concentrations above 300 units/ml decreased the level of IgG detected. Interestingly both species of recombinant human alpha interferon enhanced IgG secretion at all the concentrations tested up to 10,000 units/ml.

D) Human Experiments in vivo

In contrast to the thorough in vitro data on the immunomodulating effects of INF, there are currently no reports of interferon administered in vivo affecting in vitro or in vivo Ab responses in humans. Hopefully we will be able to shed some light on this area as we are currently investigating the in vitro effects of INF injected in vivo.

Mechanisms of Immunomodulatory Activity

The influence of INF on Ab production is obviously well documented but the mechanisms involved remain to be defined.

One of the well documented effects of INF administered in vivo is the notable decrease in the number of circulating T-lymphocytes. This reduction occurs without altering the ratio of T_H to T_S cells (75,103). Reports of INF altering the number of circulating B-lymphocytes are difficult to analyse and remain largely unresolved to date, due mainly to the different treatment regimens employed (75).

It would appear from the results obtained in investigations of isolated lymphocyte subsets (in both human and murine systems) that the B

lymphocyte is directly affected (24,29,57,66,152b). Other than noting the cell subset affected however these investigations accomplished little in terms of defining the effects of interferon at the subcellular level. Rodriguez et al. (152b) studied the production of, and response to, helper factors (HF) produced by PWM stimulated radioresistant T cells. Human alpha interferon (Sigma)(10-100 units/ml) did not enhance the production of HF by T-cells. Preincubation of B-cells with 50 and 100 units/ml of alpha-INF increased the amount of Ig secreted by these cells in response to HF and PWM. This result suggests that human alpha INF enhances the B-cell response to T-cell derived growth factor. This would explain the increased Ab response often observed following preincubation of PBMNC with INF.

Inhibition of Ig secretion by INF could result from decreased DNA and/or protein synthesis. Inhibition of either cell function would reduce the B-cell's capacity to respond to mitogen or Ag but does not explain enhancement of the response. Enhancement of the humoral immune response could possibly involve changes in soluble suppressor substances.

Interferon has reportedly induced changes in the cell membrane. Some of these changes involve increases in the number of Fc receptor for IgG (50,65b) and augmentation in the expression of class 1 major histocompability antigens (169b). Changes involved in surface structures which are intimately involved in the immune function could alter cell to cell interactions and contribute to the increases and decreases in Ab production following INF treatment. Inherant in the discussion of the mechanisms involved in the modulation of Ig production by INF are the effects observed when INF is added at different times relative to the

developing immune response. The modulating effects of INF observed change with the time course of INF treatment, suggesting that at different times INF may be affecting different cell functions.

Although the B-lymphocyte is strongly implicated as being directly affected by INF, one cannot ignore the fact both alpha and beta INF have been shown to enhance the natural killing effect of human PBMNC (91) and enhance the activity of cytotoxic T-cells (207). Other similar investigations (3,128) have demonstrated that INF affects the T-lymphocyte subset. For example, Heron et al. (72) demonstrated that pretreatment of PBMNC (or of highly purified T-cells alone) with human alpha INF led to a distinctly enhanced lysis of the specific target cell.

Summary

In summary one can conclude that in addition to the classical anti-viral effects of alpha and beta-INF these substances also possess immunomodulatory activities, specifically in altering the Ig secretion response in vitro. However the modulating activities of the INF preparations documented to date are not straightforward. This is not surprising considering that there are different subtypes of INF and that the different subtypes may possess different activities (206). Time course and concentration of INF used and the sensitizing Ag or mitogen used have all been shown to influence the results obtained.

It is obvious therefore that various modulating effects of purified alpha-INF preparations (in addition to contaminating lymphokines) will occur depending ultimately on the inherant heterogeneity of the subtypes in the sample.

We have investigated the effect of lymphoblastoid interferon (Wellferon^{R}) administered *in vivo* on *in vitro* IgG secretion and Con A induced suppression in the PBMNC obtained from a group of chronic progressive MS patients.

MATERIALS AND METHODS

A) Standard IgG Secretion Assay

i) Interferon

The interferon (Wellferon^{R}) was obtained from Burroughs Wellcome Research Laboratories. It is produced in Namalwa cells, a lymphoblastoid cell line of B-cell origin and which are currently the largest single source of interferon for clinical use (51). Sendai virus is used for induction and the crude interferon preparations are purified to greater than 80%. The Namalwa interferon consists of at least eight active components (51). It has been shown that Namalwa cells induced by Sendai virus produce both alpha and beta INF, the latter comprising approximately 10-20% of the total (38b).

ii) Patients and Controls

A total of 38 patients with the chronic progressive form of multiple sclerosis (MS) were included in the study. The average age was 44.5 $\pm$ 2 years at the time of the first test. There were 20 female and 18 male patients. In a double blind procedure 20 patients were injected subcutaneously with 5×10^{6} units of interferon (Welferon^{R}) per day and 18 received a similar volume of placebo. Both groups were chosen as to be equally represented on the basis of age, sex and the Kurtzke disability scale (88). Each week four new patients were introduced into the study and blood was drawn before the injection of INF and again 7 days after the first injection. With each new group of patients entered into the study, one healthy control volunteer was also studied to determine the baseline levels of response.

Patients' blood was obtained with their consent and used for both the IgG secretion assay and the ConA induced suppressor assay.

iii) MNC Isolation

Whole blood was drawn from the antecubital veins of consenting individuals into vacutainer tubes containing preservative free heparin sodium. The blood was diluted 1:1 with Hank's balance salt solution (no Mg++, no Ca++) (HBSS) (Gibco, Grand Island N.Y.) and underlayed with ficoll-hypaque (Pharamcia Fine Chemicals Piscataway, New Jersey). The mononuclear cells (PBMNC) were subsequently isolated by density gradient centrifugation according to the method of Boyum (26). Once isolated the PBMNC were washed at least 4 x in cold HBSS and once in cold standard culture media which consisted of RPMI 1640 with 10% fetal calf serum (Flow Labs), 0.1 mg/ml gentamycine, 2 mM L-glutamine and the pH adjusted with sodium bicarbonate. The cells were subsequently resuspended to 1 x 10^6 cells per ml of culture media.

iv) MNC Cultures

Cell cultures were set up in 1 ml volumes in 12 x 75 mm plastic Falcon tubes with or without PWM (Gibco) at a final dilution of 1:300. The cultures were kept at 37°C with 5% CO_2 in air in a humidified incubator for 7 days at which time the cell free supernatants were harvested and stored at -90°C until assayed for IgG content.

v) ELISA Assay to Quantitate IgG

The IgG content of the supernatants was measured by an ELISA assay modified from that developed by Voller and Bidwell (195b).

Ninety six well polyvinyl "Immulon" microtitre plates (Dynatech) were pre-coated with 110 uls/well of affinity purified goat anti-human IgG (Cappel) gamma chain specific (10 ug/ml) in a carbonate-bicarbonate buffer (pH 9.6) at $4^{o}C$ at least overnight. (For an appendix of the buffers and washing solutions refer to ref 195b). The plates were washed free of excess Ab with PBS and 0.05% Tween 20 (v/v) and any remaining solution was discarded by flicking the plates and tapping them solidly on paper towel. To prevent non-specific binding, a blocking solution of 110 ul of PBS with 0.1% BSA was added to each well. After 1-2 hr the plates were washed as described above and the culture supernatants and standards were added to the appropriate wells. The standards consisted of human IgG serially diluted 2 fold, from 1,000 ng/ml to 1.95 ng/ml with RPMI and 0.1% BSA. The culture supernatants were also diluted with RPMI (0.1% BSA) so as to generate values which correspond to the most accurate portion of the standard curve. These samples were left in the wells for 1 hr at room temperature. The plates were then washed and 110 ul of alkaline phosphatase conjugated goat-anti-human IgG (1:1,000 dilution of stock, Tago) was added to each well for 1 hr in the dark. The substrate, p-nitrophenyl phosphate (Sigma 104-105), dissolved in a diethanolamine buffer was then added to the plates. The color developed at room temperature and after approximately 45 min the absorbance (405 nm) was read on a multi-well spectrophotometer (Dynatech MR 600) which was interfaced with an Apple^{R} Computer and printer. The level of absorbance is proportional to the amount of human IgG bound to the plates thus allowing for the construction of the standard curve. The computer, with the readings (done in triplicates) from each well, is programmed to

display and print the standard curve and subsequently derive the unknown IgG concentrations from the standard curve.

B) Con A Induced Suppressor Assay

i) Suppressor Generating Phase

Note: This assay was conducted on the same 38 patients as above. The patients' PBMNC were assayed before treatment and again after 7 days of either interferon or placebo injections. The mononuclear cells were isolated, washed and resuspended to 1×10^6 cells/ml in the same culture medium as in the IgG secretion assay.

At this point the cells were stimulated with 3 ug/ml concanavalin A (Con A) in tissue culture flasks (Falcon) stored upright at 37^oC with 5% CO_2 in air. These cells will subsequently be referred to a "S" cells for "suppressor". Control or "C" cells are set up in an identical fashion without Con A. After a period of three days both cultures (ie "S" and "C" cells) were treated with mitomycin C (50 ug/ml, Sigma) for 30 min at 37^oC. This period is referred to as the "suppressor generating" phase of the assay.

ii) Responder Phase

After the mitomycin C treatment the cells were washed free of this substance and resuspended to 1×10^6 viable cells per ml of standard culture media. To the appropriate wells of a round bottom micro-titre plate (Limbro), 1×10^5 (100 uls) "S" or "C" cells were added along with 1×10^5 fresh allogeneic untreated responder cells or "R" cells. The responder cells were then stimulated with Con A (3 ug/ml) for 3 days

at 37°C with 5% CO_2 in air. This period is referred to as the "responder phase". At the end of the responder phase the cells were pulsed for 6 hr with 1 uCi/well of tritiated thymidine. The cells were then harvested onto glass fibre filters via a home made cell harvester and dried. Scintillation fluid was added and the vials were counted in a scintillation counter (LS 9,000, Becton Dickenson). The results were expressed as counts per minute (CPM).

This assay measures the non-specific suppression induced by Con A stimulated PBMNC on the proliferative response of fresh heterologous PBMNC stimulated with Con A. The suppression thus generated is calculated as a percentage based on the difference in CPM between the suppressor culture ("S" cells + "R" cells) and the control cultures ("C" cells + "R"-cells) using the following formula

$$\% \text{ Suppression} = 1 - \left[\frac{S(- \text{ background}) + R(- \text{ background}) + \text{Con A}}{C(- \text{ background}) + R(- \text{ background}) + \text{Con A}}\right] \times 100$$

C) IgG Secretion in Cell Subset Reconstitution Assays

i) Plastic-adhering Cell (monocyte, macrophage) Reconstitution .

a) Donors.

Donors included 6 MS patients, 3 of which were treated with Wellferon^R (5 x 10^6 u/day for at least 1 month) and 3 of which were treated with placebo. The patients were not informed of which treatment they were receiving.

PBMNC were isolated as previously described, washed 3x in cold HBSS, 1x in cold culture media, and resuspended to 2 x 10^6 cells per ml of culture media.

b) Monocyte Isolation

The monocytes were separated from the mononuclear cells by virtue of their plastic adhering properties. Twenty million PBMNC were added to 25 cm^2 tissue culture flasks (Falcon) and placed horizontally at 37^oC for one hr. After this period the non-adherent cells (NAC) were recovered and the removal of plastic-adhering cells (PAC) was repeated two more times. The non-adherant cells (NAC) (monocyte depleted PBMNC) were resuspended to 1 x 10^6 cells/ml of culture media. The plastic adhering cells, (PAC) ie. monocytes or macrophage were recovered from the first incubation by scraping the flasks with a rubber policeman; washed once and resuspended to 1 x 10^6 cells/ml. The efficacy of the PAC depletion was assessed by the inability of PAC depleted MNC to respond to PWM and also by FACS analysis.

FACS IV (Fluorescent Activated Cell Sorter) Analysis :

For routine analysis 1 x 10^6 PBMNC were labelled with a saturating amount of murine monoclonal antibody conjugated with fluoresceine isothiocyanate for 30 min on ice. The cells were subsequently washed three times in cold Hank's balanced salt solution and run through the FACS IV (Becton Dickenson). Ten thousand cells are analyzed from within the predetermined lymphocyte scatter peak for the amount of fluorescence. Florescence is expressed as percent positive fluorescence by subtracting the fluorescence of the control cells.

c) Cultures.

The following cultures were set up. INF treated patients' NAC reconstituted with 20% INF treated patients' PAC or with 20% of the

placebo treated patients' PAC and also the cultures of placebo patients' NAC reconstituted with either INF treated PAC or placebo treated PAC.

The cultures were set up in duplicate 1 ml volumes in 12 x 75 mm Falcon culture tubes. One culture received PWM (1:300 dilution) the other received only culture media. The negative control consisted of unreconstituted monocyte depleted PBMNC cultures. After 7 days of incubation supernatants were harvested as described earlier.

ii) T-helper (T_H) cell Reconstitution Assay.

a) Donors

Donors consisted of 4 MS patients, two of which had received Wellferon^R interferon injections for at least 1 month and of two which had received placebo. The PBMNC were isolated and washed as previously discussed.

b) T-B lymphocyte separation; E-rosette separation

T-cells were separated from the PBMNC by E-rosette separation. Briefly PBMNC, 1 x 10^7/ml, were mixed with a 1% solution (v/v) of packed neuraminidase treated SRBC (30 min at 37^{o}C with 0.75% v/v neuraminidase). These cells were thoroughly mixed, then allowed to incubate at 37^{o}C for 15 min, spun at 1,000 RPM for 10 min and then further incubated for an hour at 4^{o}C. The rosetted cells (ie PBMNC bound by SRBC) were then centrifuged through ficoll hypaque at 400 X g for 30 min..

The cells at the interface are referred to as E^- (ie. not binding sheep erythrocytes) and are composed mainly of B-cells and monocytes. Contaminating T-cells were removed from this population by repeating the E rosette procedure.

The pellet (after the ficoll hypaque density centrifugation step) contains the cells referred to as E^+ (ie. positive for binding sheep erythrocytes) and is composed mainly of T-cells and SRBC. The SRBC are removed by lysis with distilled water (the solution is rapidly brought back to an isotonic state with the proper concentration of saline), followed by 3 washes. The purity of the E^+ rosette procedure was assessed by two methods; 1, by culturing the E^- cells and E^+ cells alone and with PWM, and 2, by flow cytofluorometry using the FACS 4. (Becton Dickenson), as discussed in the previous section.

c) T_H-cell Isolation

The T_H cell subset was separated from the E^+-cell population by a modification of the panning technique employed by Tsoi et al. (187).

Briefly 1 x 10^7 purified T-cells (E^+-cells) were incubated with 500 ul of a 1:50 dilution of the OKT8 monoclonal Ab for 30 min at 4^oC. This Ab recognizes a specific surface marker present on a subset of suppressor T-lymphocytes.

The lymphocytes treated with the Ab were washed 3x in cold HBSS and reconstituted to a volume of 3 ml. This cell suspension was added to petridishes previously coated with 160 ug of goat-anti mouse Ig and 75 ug of goat anti-human Ig (the latter removes any surface Ig positive (B-cells) cells which could have been contaminating the E^+-cells). These plates bind the cells labelled with the monoclonal Ab. After approximately 2 hours at 4^oC the non-adherent cells are recovered, washed once in cold culture media and resuspended to 1 x 10^6 cells/ml. This process removes the population of T_S cells that express the OKT8 Ag and leaves the non-adherent cells enriched for T_H-cells.

d) Culture.

Five hundred thousand E^- (B-cells and monocytes) from each patient were cultured with 5 x 10^5 T_H-enriched cells of each of the other patients and also with their autologous T_H-cells. The cell cultures were set up in duplicate one ml volumes in 12 x 75 mm plastic Falcon culture tubes. One culture was stimulated with a 1:300 dilution of stock PWM the other culture received a similar volume of RPMI and served as a control. Other controls consisted of E^- and T_H-cells cultured alone and stimulated with PWM, the absence of Ig secretion being an indication of subset purity. After 7 days at 37°C in 5% CO_2 in air the cell free supernatants were harvested and stored at -90°C until assayed.

Results

A) IgG Secretion Assay

The amount of IgG secreted by 1 x 10^6 peripheral blood mononuclear cells (PBMNC) stimulated with and without PWM was measured in 38 MS patients and 21 healthy control volunteers.

The MNC's obtained from the MS patients were examined before treatment and after one week of daily subcutaneous injections of either interferon or placebo. These results were compared with the level of IgG secreted by PWM stimulated MNC obtained from healthy control volunteers.

We first compared the mean level of IgG secreted by the PWM stimulated MNC of the 38 MS patients to the level secreted by the MNC of the 21 healthy control volunteers (See Table II).

The level of IgG secreted by the PWM stimulated MNC obtained from

the MS patients was 2373 ± 233 ng/ml (mean ± SEM, n = 38). The level of IgG secreted by the PWM stimulated MNC obtained from the control group was 1313 ± 293 ng/ml (n = 21). Using a two tailed student t-test this difference was significant ($t = 2.99$, $p < .01$,). The mean level of IgG secreted by the unstimulated MS cultures was 271 ± 42 ng/ml and the level secreted by the unstimulated control cultures was 183 ± 15 ng/ml. The difference is significant ($p < .05$, $t = 2.2$) (see Table III).

We next compared the level of IgG secreted by 1×10^6 MNC of the 38 MS patients before treatment and again after one week of daily subcutaneous injections (see Table V). The levels of IgG secreted by the MNC of the placebo and interferon groups were not different prior to treatment. (Placebo, 2542 ± 339 ng/ml n = 18 vs Interferon, 2221 ± 326 ng/ml, n = 20). In the placebo treated group one week of daily subcutaneous injections did not significantly alter the amount IgG secreted, (2542 ± 339 ng/ml before, 2176 ± 419 ng/ml after).

However the level of IgG secreted by the MNC isolated from the 20 interferon treated patients was dramatically reduced after 1 week of daily subcutaneous injections (2221 ± 326 ng/ml before, 560 ± 75 ng/ml after). The change was highly significant as assessed by the student t-test ($t=5.2$ $p < .001$).

The modulating effect of the interferon injections on PWM induced IgG secretion became very apparent if the level of response was broken down into either a high (> 1000 ng/ml) or low response (see Table VI). Prior to INF injections 17 of the 20 (85%) MS patients were high responders. After 1 week of daily injections there were only 3 of the 20 (15%) PWM stimulated MNC cultures that secreted greater than 1,000 ng/ml

of IgG. In the placebo group 15 of the 18 (83%) were high responders. After one week of injection 12 of the 18 (67%) remained high responders.

B) Con A Induced Suppressor Cell Assay

The Con A suppressor cell assay is a measure of the ability of Con A stimulated mitomycin-treated lymphocytes to suppress the proliferative response of heterologous Con A stimulated lymphocytes. This inducible suppressor cell response measured as a percent suppression was examined in the peripheral blood of 33 chronic progressive MS patients and 18 healthy controls (some of the results were discarded due to technical difficulties). The results are presented in Table VII. The mean level of suppression induced in the MS group was 7 $\pm$ 5% (mean +/- SEM) and 27 $\pm$ 4% in the control group. Using the student t-test this difference was significant ($t = 3.2$, $p < .01$). Eighteen of the 33 MS patients (54%) had no detectable suppressor cell responses (ie < 0). The level of suppression ranged from minus 65% to plus 49% suppression. In the control group 17 of the 18 (94%) persons tested had detectable suppressor cell responses ranging from 2 to 54% suppression. (For individual results see Fig. 1)

Additionally the inducible suppressor cell response was measured in the MS group one week after having received daily subcutaneous injections of either 5×10^6 units of lymphoblastoid interferon or of placebo (see Table VIII). The injections did not alter the level of suppression detected.

In the interferon treated group the level of suppression before treatment was 11% and after treatment the level of suppression induced remained 11 $\pm$ 6%.

C) IgG Secretion in MNC Subset Mixing Experiments

i) E^+ - E^- Mixing Experiment.

The PBMNC isolated from 4 MS patients (2 interferon treated, 2 placebo treated) were separated into E^- (B-cells and monocytes) and E^+ (T-cells). The cells expressing the OKT8 Ag (T-suppressor cells) were removed from the E^+ population leaving the E^+ subset enriched for T-helper cells (for purity see Table XIII).

The T-helper enriched cell subset obtained from each of the patients was mixed with the autologous E^- cell subset and each other patients' E^- cell subpopulation (ie. INF-treated patients' E^- were reconstituted with i, autologous T_H enriched subpopulation ii, in vivo INF treated heterologous T_H enriched MNC, and iii, heterologous in vivo placebo treated T_H enriched MNC. The cell mixtures were then stimulated with PWM and after seven days the amounts of IgG secreted into the supernatant were calculated (see Table IX).

The T-helper cells obtained from a high responding placebo treated patient could not support a high response (ie >1000 ng/ml IgG) when mixed with the E^- cells of interferon treated patients. Similarly T-helper cells obtained from INF treated patients could not reconstitute a significant level of PWM induced IgG secretion when mixed with interferon treated E^- subpopulation and stimulated with PWM. This result suggests that the E^- subpopulation of the interferon injected patients could not support PWM induced IgG secretion.

The T_H cells obtained from the interferon treated patients did support a high response (1077 ng/ml) when mixed with placebo treated patients E^- cells.

The purity of the cell subset separation procedures was assessed both functionally and analytically.

Functional purity was analysed by the absence of IgG secretion in purified cell subsets following incubation with PWM. None of the subsets (i.e. E^- + PWM or E^+ + PWM) when cultured alone with the mitogen secreted IgG above background levels, indicating a satisfactory separation protocol (data not shown).

The subsets were also assessed analytically by monoclonal antibody staining and processing through the fluorescent activated cell sorter (see Table XII). Greater than 85% of the cells contained in the E^+ (T-cell) subpopulation expressed the Leu-1 Ag (pan T-cell) marker and less than 7% of the cells expressed surface immunoglobulin (sIg). This subpopulation of T-cells was subject to a panning procedure which included the removal of sIg positive by goat anti-human IgA, IgG and IgM bound to the petri dish. However the purity of the final cell preparation was not analyzed due to the limited number of cells obtained.

The "panning" procedure removed greater than 80% of the OKT8+ cells (see Table XIII).

ii) Plastic Adhering Cell (PAC) and Non-Adhering Cell (NAC) Mixing Experiment

Monocytes adhere to plastic (192). The procedure yields plastic adhering cells (PAC) and non adherent cells (NAC). Our goal was to investigate whether or not the monocytes obtained from interferon treated patients differed functionally from the monocytes obtained from placebo treated patients. This was done by mixing the monocytes (PAC) with

autologous and heterologous NAC, and then measuring the level of IgG secreted in response to PWM stimulation. The results are presented in Table X.

The data presented are the results obtained from 6 MS patients, 3 treated with INF and 3 treated with a placebo.

There was no significant difference in the level of IgG secreted by the PWM stimulated NAC cells regardless of the source of the monocytes with which they were reconstituted. The levels of IgG secreted by the NAC were not different whether reconstitution was done with monocytes obtained from the interferon treated patients (1906 $\pm$ 554 ng/ml) or with monocytes obtained from the placebo treated patients (1708 $\pm$ 650 ng/ml).

The monocyte reconstituted (PAC) interferon treated NAC secreted only 581 $\pm$ 201 ng/ml of IgG in response to PWM stimulation. The placebo treated NAC on the other hand reconstituted with monocytes obtained from the same patients secreted 3252 $\pm$ 379 ng/ml of IgG in response to PWM. This difference is highly significant ($n = 12$, $t = 6.23$, $p < .001$).

It would appear that the accessory cell role of the monocyte investigated in this assay is not affected by the <u>in vivo</u> interferon injections. Additionally it appears as though the function of one of the lymphocyte subsets contained within the NAC subpopulation (T-cells or B-cells) is affected.

It has been well documented that monocytes are required for the PWM induced differentiation of resting B-lymphocytes into IgG secreting cells. The purity of the monocyte depletion was controlled by the inability of the PWM stimulated NAC cells to secrete a significant amount of IgG (See Table XI). The monocyte depleted cultures did not secrete

IgG above the background level. (Background = 282 $\pm$ 39 ng/ml, monocyte depleted MNC + PWM = 338 $\pm$ 103 ng/ml).

Table I

Monocloncal Ab to Human PBMNC Surface Antigens

Antibody	Description	Range a % of PBMNC
Anti T-1 (Ortho) Anti T-3 (Ortho)	Pan T-cell markers	75 ± 11%
Anti Leu 2a (BD) Anti OKT5 (Ortho) Anti OKT8 (Ortho)	T-cytotoxic/suppressor	26 ± 8%
Anti Leu 3 (BD) Anti T4 (Ortho)	T-helper/Inducer	45 ± 10%

a. Samples: Normal peripheral blood mononuclear cell suspension isolated by ficoll hypaque density gradient centrifugation (26).

Detection System: Indirect immunofluorescence with FACS IV analysis (scatter gates set on the lymphocytic peak). The normal range is expressed as a percent of peripheral blood lymphocytes.

Table II

IgG Secreted in Culture by MNC Stimulated with PWM (mean ± SEM)

Population	Number of Individuals	IgG Secreted (ng/ml)[a]	Range (ng/ml)
MS	38	2373 ± 233*	139-5109
Control	21	1313 ± 266	102-4504

Abbreviations: MNC-mononuclear cells, PWM-pokeweed mitogen
IgG-immunoglobulin G, SEM-standard error of the mean
* different from control population, $p < .01$, (t-test).

a. amount of IgG secreted into 1 ml by 10^6 PBMNC stimulated with a 1:300 dilution of PWM.

Table III

IgG Secreted in Culture by Unstimulated MNC Isolated from Control Subjects and Treated and Untreated MS Patients

Donor Group (and Number)	Level of IgG Secretion (ng/ml) (a)
MS (38)	271 ± 42
Control (21)	183 ± 15*
MS before PLA (18)[b]	216 ± 26
MS after PLA (18)	237 ± 30
MS before INF (20)[c]	321 ± 75
MS after INF (20)	399 ± 110

a) mean ± SEM.
b) PLA = placebo treated; daily injections of normal saline for 1 week.
c) INF = interferon treated; daily injections of 5×10^6 IU of interferon for 1 week.

* Different from MS; $t = 2.2$, $p < .05$; Two tailed student t-test.

Table IV

LEVEL OF PWM INDUCED IgG SECRETION
IN MS AND CONTROL INDIVIDUALS

	MS	CONTROL
LOW RESPONDERS[a]	6	11
HIGH RESPONDERS[b]	32	10

[a] LOW RESPONDERS - secrete less than 1000 ng/ml IgG
[b] HIGH RESPONDERS - secrete greater than 1000 ng/ml IgG
$X^2 = 7.13$; $p < .01$

Table V

Effect of in vivo Injection of Interferon on the Amount of IgG Secreted in Culture by MNC Stimulated with PWM (mean ± SEM)

Group	IgG Secreted Before Injection (ng/ml)	IgG Secreted After Injection (ng/ml)
Placebo $(18)_a$	2542 ± 339	2176 ± 419
Interferon $(20)_b$	2221 ± 326	560 ± 75**
($5x10^6$units/day 1 week)		

**diferent from interferon before injection, $t = 4.97$, $p < .001$, (t-test)

a) Placebo = placebo treated, as in Table III.

b) Interferon = interferon treated, as in Table III.

Table VI

Effect of Interferon Injection on the Level of PWM Induced IgG Secretion In PBMNC Cultures Isolated From MS Patients

	MS PATIENTS			
	INF TREATED*		PLA TREATED**	
	BEFORE	AFTER	BEFORE	AFTER
LOW RESPONDERS [a]	3[c]	17	3	6
HIGH RESPONDERS [b]	17	3	15	12

a Low Responders - MNC secreted < 1000 ng/ml in response to PWM
b High Responders - MNC secreted > 1000 ng/ml in response to PWM.
c Number of individuals whose PBMNC secreted either greater or less than 1000 ng/ml of IgG in response to PWM stimulation.
* $X^2 = 9.1$, $p < .01$
** $X^2 = .6$, $p > .1$

Table VII

Suppressor Activity in Controls and in MS Patients

Donor Group and number	Suppressor Activity		
	R Cells + Con A + C cells	R Cells + Con A + S cells	Suppresion (b) (%)
MS (33)	35523 ± 1700(a)	34305 ± 2434	7 ± 5
Control (18)	35423 ± 2581	28582 ± 2796	25 ± 4[c]

a) CPM ± SEM
b) Mean ± SEM, Percent Suppression =

$$1 - \frac{\text{Mean CPM of R cells + Con A + S cells}}{\text{Mean CPM of R cells + Con A + C Cells}} \times 100$$

c) Different from MS; $t = 2.81$, $p < .01$

Fig. 1 Concanavalin A induced suppressor cell response in 33 chronic progressive multiple sclerosis patients and 18 healthy controls. The median is indicated for both groups. The results are expressed as percent suppression calculated from the following formula:

$$\% S = 1 - \left[\frac{\text{mean CPM of R cells + S cells + Con A}}{\text{mean CPM of R cells + C cells + Con A}}\right] \times 100$$

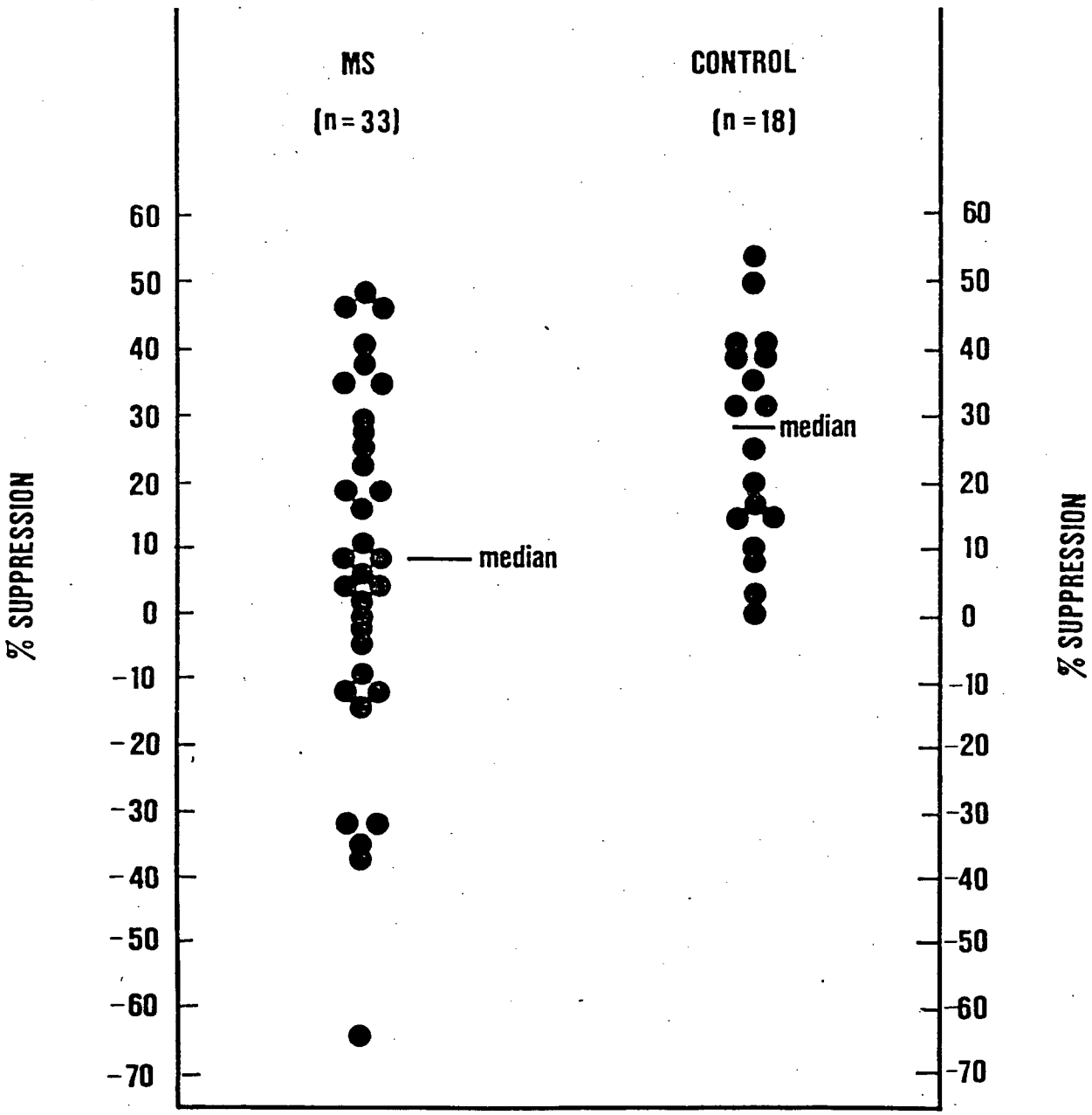
MS
(n = 33)
CONTROL
(n = 18)
median
median
% SUPPRESSION
% SUPPRESSION
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70

Table VIII

Con A Induced Suppressor Activity in MS Patients Before Treatment and After 1 week of Daily Injections of Interferon[a] or Placebo[b]

Donor Group and Number	Suppressor Activity		
	"R" Cells + Con A + "C" Cells	"R" Cells + Con A + "S" Cells	Suppression (%)
INF before (18)	34719 ± 2195(C)	32136 ± 2809	11 ± 6 (d)
INF after (18)	30651 ± 1874	26859 ± 2316	11 ± 6
PLA before (15)	37467 ± 2831	39490 ± 4245	-4 ± 7
PLA after (15)[e]	35825 ± 2572	34965 ± 3414	2 ± 6

a) INF = In vivo injections of 5×10^6 units of lymphoblastoid (Wellferon ^{R}) interferon per day for 1 week.

b) PLA = In vivo injection of a similar volume of normal saline per day for 1 week.

c) CPM ± SEM

d) Mean ± SEM, for calculation of percent suppression, see Table VII.

e) Some samples were not processed due to technical difficulties, i.e. out of 38 MS patients' samples, 5 had to be discarded.

Table IX

IgG Secreted in Reconstituted MNC Cultures Stimulated With PWM: Effect of *in vivo* Interferon Injection on the T_H and B-lymphocyte Subpopulations (mean ± SEM)

Source of E^--cells (B-cells, monocytes)	Source of T_H-cells	IgG Secreted (ng/ml)	Range (ng/ml)
Interferon*	Interferon	316 ± 51[b]	176-419
Interferon**	Placebo	283 ± 32	244-344
Placebo	Interferon	506 ± 202	212-1077
Placebo	Placebo	988 ± 489	273-2431

a 5×10^5 E^- were mixed with 5×10^5 TH in 1 ml of culture medium and stimulated with PWM (1:300 final dilution).

b Each result is the mean of 2 reconstitution experiments.

* [INF E^- + INF T_M] vs [PLA E^- + INF T_M]; $t = .9$, $p < .05$

** [INF E^- + PLA T_M] vs [PLA E^- + PLA T_M]; $t = 1.4$, $p < .05$

Table X

IgG Secretion in Reconstituted MNC Cultures Stimulated with PWM: Effect of *in vivo* Interferon Injection on NAC and PAC (mean ± SEM)

Source of NAC	Source of PAC	IgG Secreted (ng/ml)
PLA NAC	INF - PAC	3492 ± 434[b]
PLA NAC	PLA - PAC	2931 ± 727
INF NAC	INF - PAC	637 ± 291
INF NAC	PLA - PAC	486 ± 298

Abbreviations: IgG-immunoglobulin-G, MNC-mononuclear cells, PWM-pokeweed mitogen, PLA-NAC-placebo treated non-adherant cells
PLA-PAC- " " " plastic adherent cells
INF-PAC interferon treated plastic adherent cells

[a] - 4×10^5 non-adherent cells (NAC) were mixed with 1×10^5 plastic adhering cells (PAC) in 1 ml of culture medium and stimulated with PWM (1:300 final dilution)

[b] - Results are expressed as the mean ± SEM of the amount IgG secreted into the culture supernatant.

[c] - Each result is the mean of three reconstitution experiments.

Fig. 2 The level of Con A induced suppression vs the amount of PWM induced IgG secretion in the PBMNC isolated from the same person. The results of 12 MS patients and 12 controls are included. Note there is no correlation between these two in vitro immunological parameters even though the level of response obtained in each assay is clearly influenced by a common lymphocyte subset, namely the OKT8 suppressor cells.

Note: Level of IgG vs %Con A Suppression, Correlation Coefficients, r.

MS r = .36

Control r = -.02

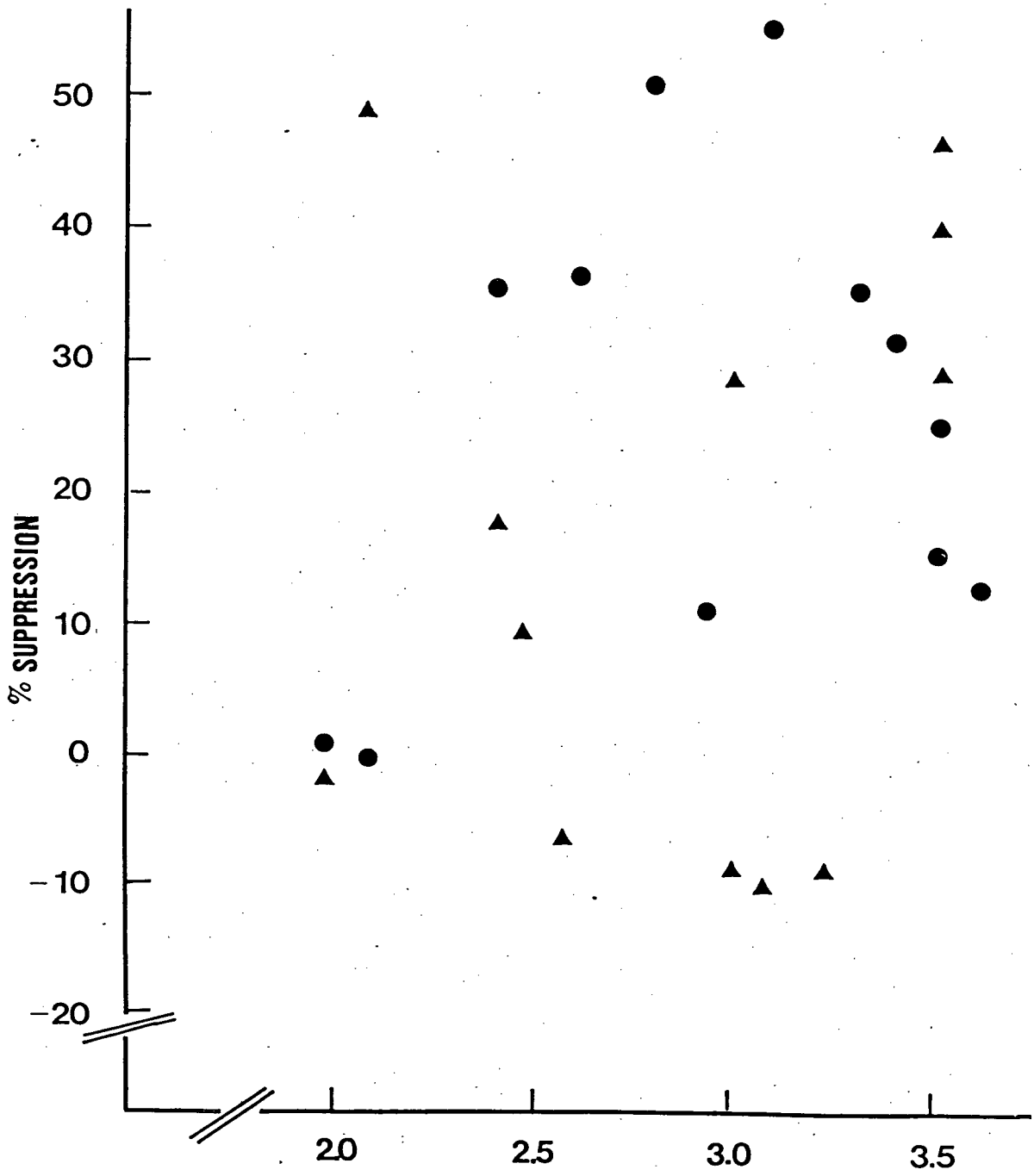
50
40
30
20
10
0
−10
−20
% SUPPRESSION
2.0
2.5
3.0
3.5
Log10 LEVEL OF IgG SECRETED (ng/ml)
▲- MS
●- CONTROL

Table XI

IgG Secreted by PWM Stimulated Cultures of MNC and Monocyte Depleted MNC

Donor and Treatment	MNC with no mitogen	MNC + PWM	Monocyte Depleted MNC (NAC) + PWM
1. INF	304 ± 17	6377 ± 1992	149 ± 3
2. INF	182 ± 2	165 ± 7	351 ± 8
3. INF	319 ± 13	284 ± 7	237 ± 1
4. PLA	273 ± 6	4060 ± 890	776 ± 242
5. PLA	450 ± 24	3783 ± 363	177 ± 4
6. PLA	163 ± 1	157 ± 3	NA
Mean ± SEM	282 ± 39	2471 ± 985	338 ± 103

One million were incubated alone or with PWM (1:300 final dilution); 1 x 10^6 monocyte depleted MNC (NAC) were also cultured with PWM.

Table XII

Purity of B, T Cell Separation

Exp #	Cell Subset	% Leu-1 +	%+ve sIg (Rabbit antihuman IgG,A,M)
1	E^+ (T-cells)	90	7
	E^- (B-cells, monocytes)	4	80
	MNC	75	22
2	E^+	90	6
	E^-	7	80
	MNC	75	16

One million cells from each subset were incubated with a 1:100 dilution of Leu-1 FITC (50 ul) and a 1:50 dilution (50 ul) of rabbit anti-human IgG,M,A - FITC for 30 minutes on ice. The cells were analyzed on the FACS. The percent fluorescence was assessed by subtracting the fluorescence of the negative control cells from the test samples. The scatter gates were set on the lymphocyte peak.

Table XIII

Efficiency of OKT8 Removal

Cell Subset	% OKT8	
MNC	25.1	
MNC minus OKT8+ cells	4.4	83% of the T8 positive cells were removed
OKT8+ cells	67.0	

The cells bearing the OKT8 marker were removed from the MNC by a panning procedure. Briefly the MNC (1×10^7) were incubated on ice with 500 ul of a 1:50 dilution of OKT8. The cells were then washed free of non-binding OKT8 and added to petri dishes pre-coated with goat anti-mouse IgG. The non-adherent cells were then recovered and stained with a 1:50 dilution of goat anti-mouse IgG labelled with fluorescein. The percent positive cells were analyzed on the FACS as discussed above.

Discussion

We have measured the levels of IgG secreted by 10^6 PWM stimulated peripheral blood mononuclear cells obtained from chronic progressive, multiple sclerosis patients and normal subjects. The MNC isolated from the MS group secreted significantly more IgG in response to PWM ($p < .01$) than did the MNC of the control group. This observation has previously been documented (10,62,123,178). Several investigators have studied the underlying cause of this elevated, humural immune response, and have noted reduced suppressor activity (62,82) enhanced T-helper cell activity (93) as well as altered B-lymphocyte function (62,93).

It has also been documented that the number of lymphocytes expressing suppressor functions is reduced during active disease. Thymus derived lymphocytes (T-cells) that bear the surface receptors for the Fc fragment of IgG (T_G-cells) can upon interaction with IgG immune complexes suppress the PWM induced differentiation of the B-lymphocyte (114). Huddlestone and Oldstone's group observed that the percentage of T_G cells is reduced during the active course of the disease (76). Using monoclonal Ab's specific for cell-surface markers present on T-suppressor lymphocytes (namely OKT5+ and OKT8+) (142,146), many investigators have noted a decrease in the porportion of MNC bearing these surface markers during active disease (18,130,146,136).

Although the absolute number of OKT8+ cells added to in vitro cultures clearly influences IgG secretion, there appears to be no correlation between the proportion of OKT8+ cells within ficoll-hypaque purified MNC and the level of PWM induced IgG secreted by these cells for both MS patients and controls (10). So although as a group the MNC

isolated from MS patients exhibit elevated levels of PWM induced IgG secretion and decreased levels of cells expressing the suppressor phenotype, there appears to be no correlation in individual patients between these two parameters.

Analysis of the response of PWM stimulated PBMNC have revealed large fluctuations in the level of response (10,69,83,156). Individual patients reproducibly secrete either high or low amounts of IgG in response to PWM (defined as < or > 1000 ng/ml IgG secreted by 1 x 10^6 MNC/ml). In our investigation involving 38 MS patients and 21 controls we found that in the MS group 84% were high responders as compared to 48% in the normal control group (Table IV).

In a series of cell subset mixing experiments which were designed to investigate the regulatory T_H and T_S cell subsets involved in the PWM induced differentiation of B-lymphocytes, the group in Chicago (156) observed that the OKT8+ cells isolated from a low responding individual suppressed the PWM induced differentiation of a pool of B and T_H lymphocytes to a much greater extent that did the same number of OKT8+ cells isolated from a high responder.

This observation suggests that the functional state of the OKT8 lymphocytes may be altered without a concomittant alteration in the number of cells. This would explain why there is no correlation between the number of OKT8+ cells and the level of PWM induced IgG secretion observed in individual patients and controls. Additionally there is an over representation of high responding individuals within the MS population suggesting that there may be an alteration in the functional state of their OKT8+ lymphocytes.

In a more direct manner we have studied the suppressor function in these MS patients; a non-specific inducible suppressor cell assay. This assay measures the ability of Con A, mitomycin C treated MNC to suppress the Con A stimulated proliferative response of heterologous thymus-derived lymphocytes (T-cells). The level of suppression induced in the MNC isolated from the MS patients was significantly lower ($p < .01$) than the level induced in the control MNC. There was no negative suppression observed in the control group whereas 33% of the MS patients PBMNC showed a negative suppressor response. We have been unable to determine the underlying cause of this phenomenon. Reduced Con A induced suppression has been noted by several groups (61,118,203). Other assays of T-suppressor function have also shown decreased suppressor cell activity during attacks of MS (6,13,61,118,198).

The level of suppression observed in individual cultures did not correlate with the level of IgG secreted and this was the same for both the control and MS group (fig. 2).

This observation is paradoxical for the following reasons. It is documented that the number of T-suppressor cells is reduced in active MS. Reinforcing this observation is the reduced suppresor cell activity and increased T-cell dependent IgG secretion that is observed <u>in vitro</u> in the MNC isolated from MS patients with active disease.

However what appears to be a simple phenomenon is actually quite complex because there is no correlation between the proportion of OKT8+ cells in ficoll-hypaque separated MNC's and i, the amount of PWM induced IgG secreted (10) or ii, the level of Con A induced suppression. Additionally the level of IgG secreted by PWM stimulated MNC does not

reflect the level of suppression induced by the Con A activated MNC of the same individual. This paradox exists even though it is well known that OKT8 lymphocytes are intimately involved in both the Con A (10) assay system as well as the PWM induced IgG secretion assay (10,156).

The apparent lack of correlation between lymphocyte function and numbers likely stems from the fact that the $OKT8^+$ subset is not homogeneous based either on the expression of OKT8 (177b), and other cell surface markers (137, 177b) or cell subset function ($OKT8^+$ cells contain both cytotoxic and suppressor cell functions (145, 193)). For example the OKT8 monoclonal Ab defines approximately 30% of the peripheral T-cells and the entire OKT5 + population as well as the $OKT4^-$ $OKT5^-$, T-cell subset (145). The latter lymphocyte subpopulation has not been defined by monoclonal Ab and may contain an important suppressor feedback regulator population (145). The precise role of this feedback regulator population as well as its regulatory mechanisms remain to be defined. It seems therefore that further investigation of the T-suppressor cell subset is required before any correlations can be made between the quantitative pathology and the functional abnormalities that are observed in active MS.

The MS patients discussed earlier were patients involved in a clinical interferon trial at the MS clinic in the Health Sciences Centre Hospital. The MNC's of each patient were assessed in two <u>in vitro</u> immunological assays prior to the study and again 7 days after receiving daily subcutaneous injections of either 5×10^6 units of lymphoblastoid interferon or a similar volume of placebo. Both we and the patients were unaware of the treatment until after the results were calculated.

The *in vivo* interferon treatment had a profound effect on the *in vitro* IgG secretion assay. The mean level of IgG secreted by 10^6 PWM stimulated MNC was reduced from 2,221 ± 325 ng/ml to 560 ± 75 ng/ml (p .001) in the 20 patients studied. The amount of IgG secreted by the PWM stimulated MNC isolated from 18 placebo treated did not change significantly after 1 week of daily injections (2542 ng/ml before and 2176 ng/ml after).

In this assay there is a very wide range in the level of response obtained in both normal and MS patients. In any given individual however the variation in the level of response over a fairly long period of time is much less than the variations observed in any given group (83).

Prior to injection, 85% of the patients in the interferon treated group, were "high" responders (i.e. >1000 ng/ml). After 1 week of daily subcutaneous injection only 15% of the patients remained high responders (In the placebo group 83% were high responders prior to any injection and 67% were high responders after 1 week of daily injections of saline). We conclude that the *in vivo* interferon injections had a dramatic effect on this *in vitro* humoral immune response.

We also studied the Con A induced suppressor cell response in these two groups of patients before and after injection of either interferon or placebo. There was no change in the level of Con A inducible suppression in either the placebo or interferon treated patients after one week of daily subcutaneous injections.

It has been suggested that the OKT8+ cell subpopulation contains the suppressor activity induced by Con A (10). As mentioned earlier the functional state of this OKT8+ subpopulation has also been implicated in

regulating the level of IgG secreted by PWM stimulated MNC (156). Since the in vivo treatment had no effect on the in vitro Con A induced suppressor cell assay and marked effect on the PWM induced IgG secretion assay we would suggest that the interferon was not modulating the activity of this suppressor cell subpopulation.

Which subset(s) was/were affected by the in vivo interferon injection? We investigated this question by comparing the functional activity of interferon and placebo treated B-cells, T-cells and monocytes in various cell subset mixing experiments. We compared the functional activity of E^- cells (B lymphocytes and monocytes) isolated from interferon treated patients with the activity of E^- cells isolated from placebo treated patients in their ability to reconstitute an in vitro humoral response when mixed with the T_H cells isolated from both placebo and inteferon treated patients. The E^- cells obtained from the interferon treated patients could not generate a response greater than 419 ng/ml. On the other hand the E^- cells obtained from the placebo treated patients secreted greater than 1000 ng/ml when mixed with either the interferon or placebo treated T_H cells.

This suggests that perhaps the B-lymphocyte function may have been directly altered by interferon. The E^- cell subpopulation is composed of both B-cells and monocytes so the function of the latter subpopulation was examined. Monocytes isolated from INF treated patients were compared with those isolated from placebo treated patients in their ability to reconstitute an in vitro humoral response when mixed with MNC that had previously been deprived of monocytes (i.e. NAC) both from INF and PLA treated patients.

No difference was found in the level of response between the monocyte depleted MNC (i.e. NAC) reconstituted with either placebo treated or with interferon treated monocytes (i.e. PAC).

To summarize: INF treated E^- cells did not secrete Ig when INF treated macrophage were functionally intact. This would suggest that an alteration in the B-lymphocyte contributed to the observed decrease in the level of IgG secreted by PBMNC in response to PWM.

The level of response reconstituted with placebo treated T_H cells was not significantly higher than that obtained with interferon treated T_H cells, however we cannot exclude the possibility that INF also affects the T_H cell.

This is the first report of INF administration <u>in vivo</u> affecting the humoral immune response <u>in vitro</u> in humans. Harfast et al (66) and Rodriguez et al. previously reported that interferon added <u>in vitro</u> modulated PWM induced IgG secretion. They observed that the presence of 100-5,000 units of human leukocyte interferon present throughout the 7 day culture period significantly reduced the amount of IgG secreted in culture in response to PWM. This result is in agreement with Parker's group (128) who noted a suppressed PFC response when human leukocyte interferon was added simultaneously with the Ag (horse red blood cells). In contrast to these results pretreatment of the MNC prior to mitogen or antigen stimulation resulted in a significantly enhanced IgG secretion compared to untreated controls (66,128). Harfast's group noted that the increased IgG secretion was the direct effect of the interferon pretreatment on the B-lymphocyte subset. Shalaby's group (166) similarly investigated the effects of three bacteria-derived human recombinant

alpha interferon subtypes on a specific in vitro humoral immune response. All the interferons tested enhanced the PFC formation when present throughout the entire 9 day culture period in contrast to the previous reports where the presence of interferon throughout the culture period resulted in either decreased Ig secretion (66) or a decrease in the number of specific antibody PFC (166). Another group investigating different subtypes of recombinant human interferons (206) observed that different interferon subtypes had inhibitory and enhancing effects on the level of IgG secreted by human MNC. They noted additionally that the concentration of the different INF subtypes influenced their immunomodulatory activity.

It is not surprising therefore that conflicting results are obtained between different laboratories using different interferon preparations at varying concentrations and who additionally use different Ag or mitogen. What is important is that interferon appears to have immune modulatory effects and additionally that the different subtypes and concentrations used appear to have different properties in modulating immune responses. This particular preparation of interferon appears to reduce IgG secretion measured in vitro and as such may be useful in diseases where excessive amounts of pathogenic antibodies are secreted.

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