Modification of the *in Vitro* Response to Phytohemagglutinin of Mouse Spleen Cells by Amyloidogenic Agents* (33901)

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Animals and man with impaired immunological responsiveness frequently develop generalized amyloidosis (1–4). The mechanisms by which these animals are rendered more susceptible to this disease are not known, but common features to all these animal models are in intense proliferative response of the reticuloendothelial system (5), lymphopenia (2, 6) and functional deficiencies of the thymic-dependent, cellular immune mechanisms (1).

The most commonly employed methods for the study of experimental amyloidosis in mice are the repeated subcutaneous injection of casein or azocasein (7, 8) and the intraperitoneal injection of complete Freund adjuvant (CFA) (9). These agents similarly produce proliferation of the reticuloendothelial system and according to Druet and Janigan (10), produce thymic atrophy and peripheral lymphopenia. Few studies have reported the effect of these agents on cellular immune function (11). Closely correlated with the integrity of the thymic-dependent immunologic functions is the capacity of peripheral lymphoid cells to respone *in vitro* to phytohemagglutinin (PHA), a mitogen derived from *Phaseolus vulgaris* (12). The PHA responses are regularly suppressed following neonatal thymectomy in some animals as are the cellular immunities; and patients who fail to develop the thymus-dependent immunological functions lack normal responses to PHA (13, 14).

In order to determine whether alterations of cellular immune function occur in response to casein, azocasein, or CFA, we have studied the effect of single and multiple injections of these substances on the *in vitro* response of mouse spleen cells to PHA. Our findings indicate that this response is diminished by azocasein and CFA.

Materials and Methods. Ninty-eight adult C3H mice of both sexes were used in a total of nine separate experiments. Different groups of mice received either single or multiple injections of casein, azocasein, or CFA. The casein solutions were prepared according to a method described by Janigan and Druet (15), and a single injection consisted of 0.3 ml of a 10% casein solution given subcutaneously. Azocasein (8) was given in the same concentration and dosage. The CFA was injected as a single intraperitoneal injection of 0.25 ml. Animals were killed at different periods following the initial injection and the spleens were removed aseptically and placed in culture medium.

The *in vitro* response to PHA by these tissues was studied, with some modifications, according to the method described by Lindahl-Kiessling and Peterson (16). The spleens were homogenized in Ten-Broek homogenizers in Eagles minimal essential medium containing 5% fresh or fresh-frozen, heat-inactivated human serum, 1% 200 m*M* L-glutamine (Gibco) and 100 units of penicillin and 100 μg of streptomycin /ml of medium. Nucleated cells which excluded trypan blue were enumerated on a hemocytometer and the final cell suspension was adjusted to a volume containing 2.5×10^6 cells/ml. Six culture tubes (16×100 mm), each containing 2 ml of the cell suspension, were prepared from each pooled cell suspension. Ten μl of freshly reconstituted PHA-P (Difco) were added to half of the cultures prior to incubation. All cultures were incubated at 37° in an atmosphere of 5% CO_2 in air at 100% humidity. After varying periods of incubation, ranging from 0–43 hr, 2 μCi of tritiated thymidine (New England Nuclear) sp

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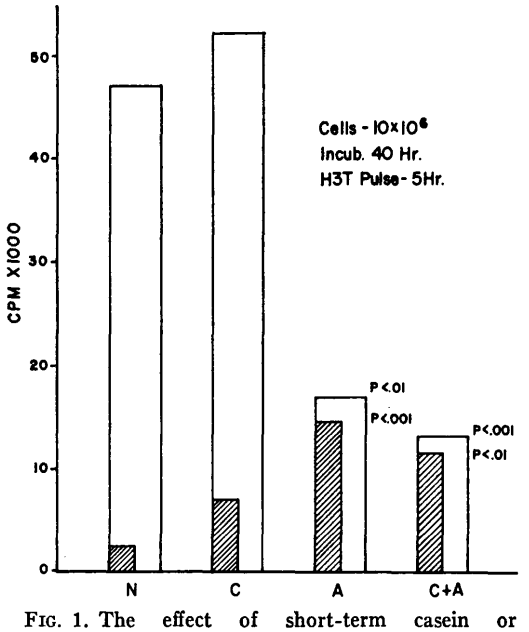

FIG. 1. The effect of short-term casein or azocasein injections on the *in vitro* response of mouse spleen cells to PHA: each bar represents the mean of triplicate tests performed on six mice; (open bars), values from PHA-stimulated cultures, and (hatched bars), from unstimulated cultures; N = untreated mice; C = casein-treated mice; A = azocasein-treated mice; C + A = mice treated with both casein and azocasein.

act 6.7 C/mmole was added to the cultures. These tubes were then incubated for an additional 5 hr. The cultures were removed from the incubator and the reactions were stopped by the addition of an excess of nonradioactive thymidine. Unincorporated thymidine was washed from the cells with isotonic saline and the cell buttons were lysed by freezing and thawing. Total protein and nucleic acids were precipitated with 10% trichloroacetic acid and washed twice with 80% ethanol. The washed precipitates were redissolved in NCS, a strong base (Nuclear Chicago), and the solution was added to the scintillation fluid. Each culture was then counted for 10 min in a Packard scintillation counter. The data are expressed as the means of triplicate samples in counts per minute per culture.

In one experiment, cell cultures from treated and untreated animals were prepared in a similar manner, with the exception that azocasein, 0.1, 1.0, or 2.5 mg was added to the cultures instead of PHA-P.

Results. The response to PHA *in vitro* is compared in mice receiving 4 daily injections of casein, azocasein, or both (Fig. 1). The uptake of tritiated thymidine was measured after 48 hr of incubation. No significant differences were observed between untreated and casein-treated animals. Azocasein, however, markedly reduced the response to PHA. Furthermore, unstimulated cells from the azocasein-treated animals incorporated larger quantities of tritiated thymidine than did control cultures.

The *in vitro* response to PHA was assessed at different time intervals in animals receiving a single ip injection of CFA and in animals receiving varying numbers of daily injections of azocasein (Fig. 2). Each bar represents a separate experiment and owing to daily variation in the method, numerical comparisons are not valid. The values, therefore, are expressed as a percentage increase or decrease of the treated cultures relative to matched control values. In both instances, suppression of the PHA response by these

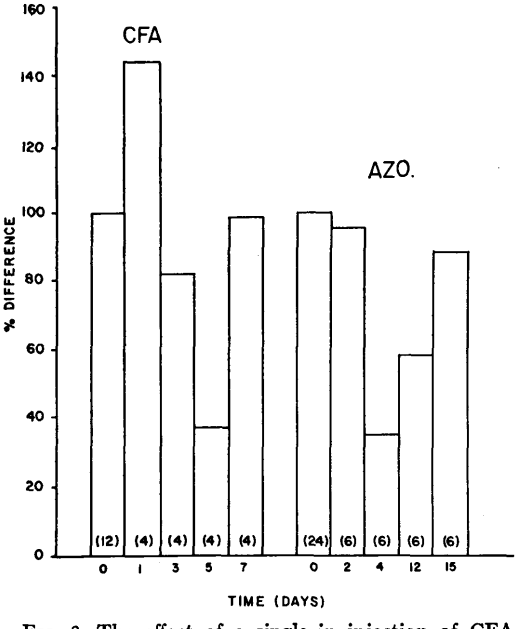

FIG. 2. The effect of a single ip injection of CFA or 4 daily sc azocasein injection on the *in vitro* response of mouse spleen cell suspensions to PHA; the number of animals per group is given in parentheses.

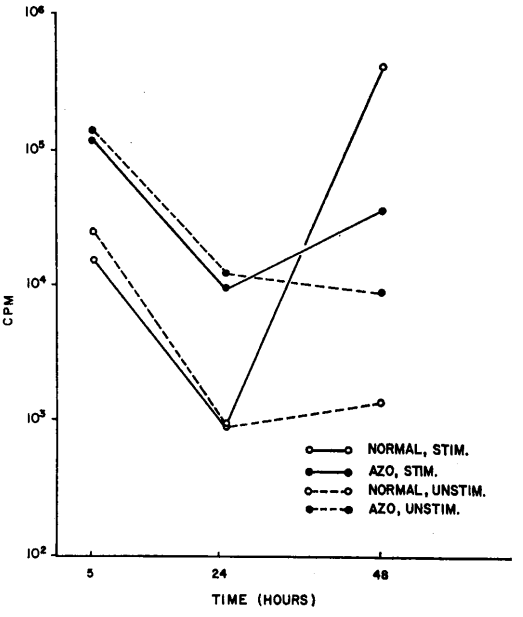

FIG. 3. The effect of 4 daily sc azocasein injections on the uptake of tritiated thymidine by mouse spleen cells stimulated *in vitro* with PHA.

compounds occurred around 4–5 days after the onset of injections. The effect, however, when 48-hr culture incubation times were used, was only transient; and the response in the CFA-treated animals is nearly normal by day 7. In the azocasein-treated group, the response is approaching that of untreated animals by day 15.

In order to better define the pattern of response in the treated animals, rates of change in DNA synthetic activity was studied in cultures incubated for 5, 24, and 48 hr. In animals treated with four daily injections of azocasein (Fig. 3), several alterations from the control animals were noted. The cultures from treated animals have higher synthetic activity at 5 hr than do untreated controls. While this activity decreases in both treated and untreated animals by 24 hr, the fall is greater in the untreated animals. Furthermore, the increase in spontaneous DNA synthesis which occurs between 24 and 48 hr is greater in the treated group.

Figure 4 shows the result of serial incubation of normal and CFA-treated animals. The tests were performed 7 days after the injection of the adjuvant, when the uptake of tritiated thymidine in the treated and untreated animals was similar. Once again, the rate of change from 24 to 48 hr suggests the response to PHA is lower in the treated group.

The high activity level at 5 hr in the treated animals suggested the possibility that these compounds might be activating the PHA-responsive cells *in vivo*. To test this possibility, spleen cell cultures were prepared from normal animals, but were stimulated *in vitro* with azocasein rather than PHA (Fig. 5). Azocasein does appear to have mitogenic activity although this is quantitatively less than that observed after PHA treatment *in vitro*.

In an effort to determine whether PHA and azocasein were stimulating the same cell populations, animals were treated *in vivo* with azocasein or PHA-P (3.0 mg ip daily) for 4 days. Spleen cell suspensions from these

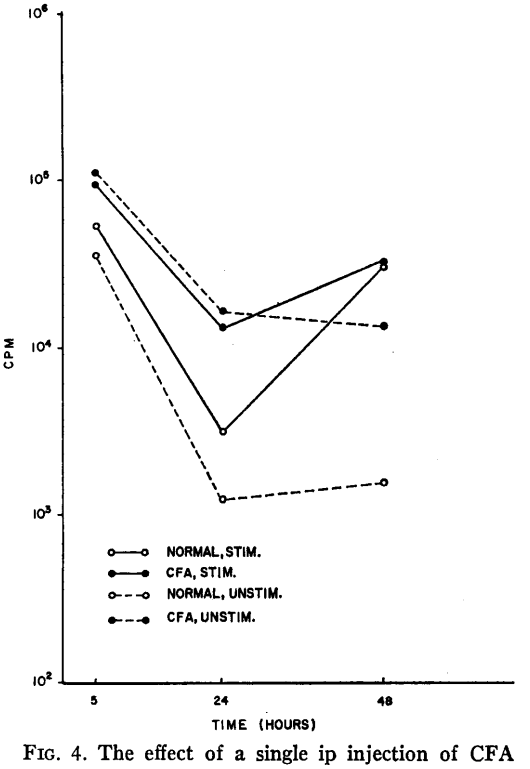

FIG. 4. The effect of a single ip injection of CFA on the uptake of tritiated thymidine by mouse spleen cells stimulated *in vitro* with PHA; the cells were obtained 7 days following the injection of CFA.

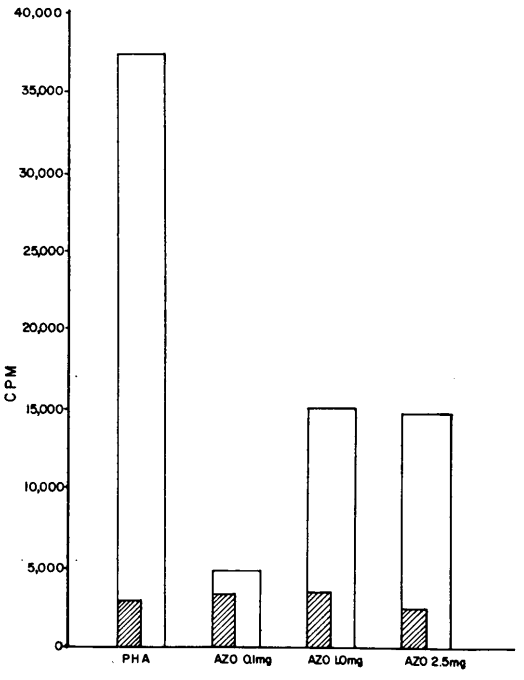

FIG. 5. The uptake of tritiated thymidine by normal mouse spleen cells cultured *in vitro* with azocasein; response to PHA is included for comparison.

animals, as well as from untreated animals, were prepared for culture and then treated *in vitro* with either PHA or azocasein (Fig. 6). *In vivo* treatment with PHA diminishes the *in vitro* response to PHA and to a lesser extent, to azocasein. *In vivo* asocasein treatment markedly reduces the response to PHA and abolished the response to azocasein treatment *in vitro*.

Discussion. There is evidence that the majority of lymphoid cells that respond to PHA are thymic-dependent (13). Our studies show that two compounds that are amyloidogenic can diminish the PHA response of mouse spleen cells *in vitro*. In treated animals, the level of DNA synthetic activity is higher at 5 and 24 hr than in cultures from untreated animals. Our data indicates that azocasein itself has mitogenic properties and that this increased activity reflects prior activation *in vivo* of potentially PHA-responsive cells. This raises a question which is difficult to answer at present; namely, does activation of this cell population *in vivo* by nonspecific mitogens represent a loss of thymic-dependent cells from a functional standpoint. If it does, then a deficiency of thymic cell function may be a common factor in the pathogenesis of amyloidosis, as has been suggested in the models where amyloidosis occurs with abnormal frequency in immunologically deficient animals. The more amyloidogenic azocasein produced clear inhibition of the PHA response whereas in the dosages and time intervals studies the less amyloidogenic casein treatment did not. It would be of interest in future studies to evaluate PHA responses and cell-mediated immunities in animals treated with casein for more prolonged periods.

Summary. The *in vitro* response to PHA by mouse spleen cells is diminished by treatment of animals with complete Freund adjuvant (0.25 ml ip) or azocasein (daily sc injection of 0.3 ml of 10% solution). The altered response appears, in part, due to prior

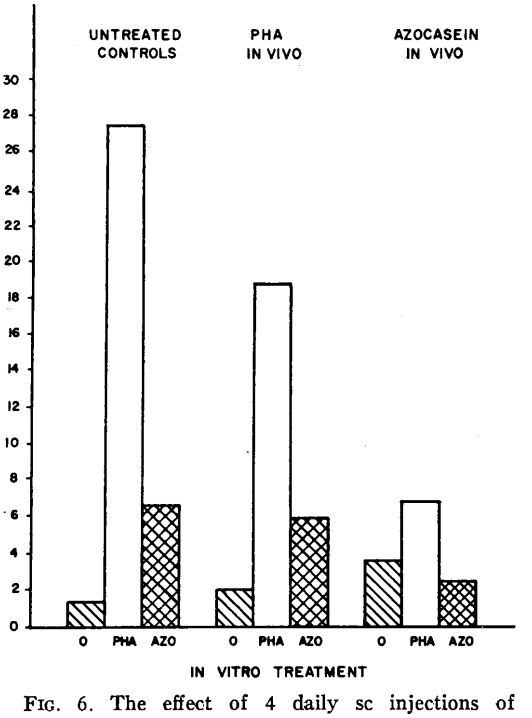

FIG. 6. The effect of 4 daily sc injections of azocasein or PHA on the *in vitro* response of mouse spleen cells to PHA or azocasein: O = untreated cell cultures; PHA = cultures incubated with PHA *in vitro*; AZO = cultures incubated with azocasein (1.0 mg/culture) *in vitro*. (cpm 1000)

in vivo activation of PHA-responsive cells by azocasein or CFA. Azocasein has some mitogenic activity when incubated with normal cells *in vitro*.

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Dissociation of Multiple Polyoma Virus Functions by Ultraviolet Irradiation (33902)

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The polyoma virus has been associated with several activities. It produces cytopathic effects in mouse embryo tissue cultures (1), produces tumors in newborn mice and hamsters (2) and transforms tissue culture cells (3), induces tumor-specific transplantation antigens (4, 5) and complement-fixing antigens (6) in these cells and agglutinates erythrocytes (7). Most of these functions have been shown to be intimately associated with each other (7–9). Recent reports, however, have suggested that some of these functions may be partially dissociated from each other (10–15).

The results of the present study indicate that the individual functions of polyoma virus can be further separated by ultraviolet irradiation. It was found that the ability of polyoma virus to affect tumor-specific transplantation resistance in mice persisted at a dose of ultraviolet irradiation which produced loss of infectivity and oncogenicity.

Materials and Methods. Dulbecco's large plaque polyoma virus, PyD, was used in these studies. The source of ultraviolet irradiation was a Westinghouse germicidal lamp, G30T8, which delivered at least 95% of energy at 2537 Å. Five ml of the virus suspension (10^6 pfu/ml) was placed in a 100-mm petri dish on a mechanical shaker, 21.2 cm from the lamp. At this distance, the energy at the surface of the petri dish was 3900 ergs/mm^2/min. Infectivity of the polyoma virus was determined by inoculating 0.1 ml of dilutions of the virus suspensions into culture tubes containing mouse embryo cells. The cultures were observed for cytopathic effect (CPE) at 2-day intervals and the tissue culture infective dose (TCID) was determined. Tumorigenicity was determined by inoculating litters of golden Syrian hamsters with 0.1 ml of virus preparations 24 hr after birth. As an assay for immunogenicity, 8-week-old C57Bl/6 JN and C3Hf/HeN mice