Growth Inhibition of Human Melanoma Cells by C-Reactive Protein (CRP) Activated Lymphocytes[1] (36322)

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While investigating rabbit immune responses to weak antigens, Wood (1) found a consistent positive correlation between the early production of rabbit acute phase protein (C_x reactive protein, C_xRP) and the subsequent production of high titers of precipitating antibody. Among the animals immunized, only those giving an early C_xRP response produced significant levels of serum antibodies. He also found (2) a correlation between C_xRP production and adjuvant effect. Noting that in various species (including man), C-reactive protein (CRP) was a sensitive indicator of inflammation, he felt the possibility also existed that CRP was directly concerned in the chain of events that lead to antibody formation. Since that time it has become clear that one of these events is the activation of lymphocytes.

We recently reported (3) that human acute phase protein (CRP) stimulated human lymphocytes *in vitro*. The present experiments were performed to determine if this stimulation altered the relationship between lymphocytes and melanoma cells in tissue culture. Melanoma cells were selected because melanoma tumor nodules can be destroyed by a local response to BCG injection. This effect seems to depend on local inflammation and stimulation of lymphocytes and coincides with the development of a cellular immune response to BCG (4). It was postulated that if CRP activated lymphocytes, it could probably induce some change in the growth rate of melanoma cells exposed to lymphocytes *in vitro*. Therefore, melanoma cells obtained at biopsy were established in culture; and their growth curve was calculated. CRP alone, lymphocytes alone, and lymphocytes plus CRP were tested to determine their effects on the standard growth curve of these melanoma cells. In addition, the lymphocyte/tumor cell relationship was studied microscopically in the presence and absence of CRP.

Materials and Methods. CRP. In order to avoid the possible introduction of extraneous material of bacterial origin, CRP was isolated by a method which did not involve precipitation of the pneumococcal C-polysaccharide. Briefly, 750 ml of CRP positive serum was obtained from patients 24–48 hr following elective surgery. Serum samples before surgery were negative for CRP; and none of the patients had infections or malignancies. The serum was diluted 4.5 times in cold pyrogen-free distilled water at pH 5.6. A precipitate formed immediately and was promptly collected by centrifugation; washed three times in distilled water at pH 5.6, and redissolved in 0.2 *M* EDTA at pH 8.6. Solid sterile ammonium sulfate was added to the final concentration of 1.5 *M*, the precipitate was removed and the supernate was dialyzed against phosphate buffered saline (pH 7.4) to remove sulfate. Gel filtration on Sephadex G200 separated CRP from most normal serum proteins and residual traces of albumin were removed by electrophoresis as previously described (3). The preparation was finally dialyzed extensively against Roswell Park Memorial Institute medium 1640 (RPMI 1640), filtered in a Millipore filter (0.2 μ pore size), tested for sterility and the protein concentration adjusted to 1 mg/ml. Recovery was estimated at 45 mg of CRP.

Melanoma cells. A lung biopsy of melanoma tissue was established in culture without

[1] This work was supported by a National Science Foundation Research Grant GB26646.

[2] RCDA Awardee, NIH.

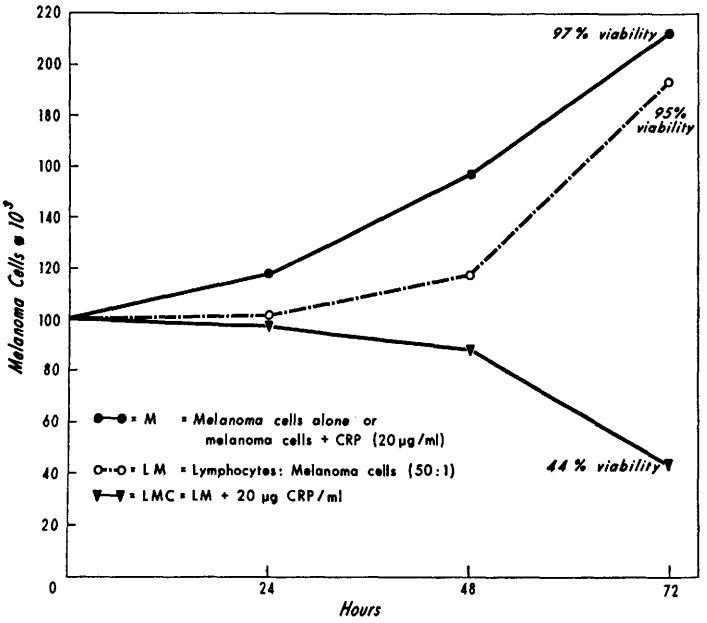

FIG. 1. Growth inhibition of melanoma cells by C-reactive protein (CRP) activated lymphocytes.

the use of mitogenic agents. The cultured cells retained the ability to produce pigment; and the histocompatibility antigens of the cell donor were known.

Lymphocytes. Lymphocytes were obtained from healthy volunteers of known histocompatibility type by the method of Colley and De Witt (5). The lymphocyte preparations contained >95% lymphocytes which were 95% viable.

Culture medium. All experiments were conducted with cells grown in RPMI 1640 with added 15% fetal calf serum.

Growth curve studies. Plastic bottles were charged with 4 ml of a suspension of melanoma cells at a concentration of 2.5×10^4/ml and incubated at 37° in 5% CO_2. The growth curve was determined by counts of sequential samples. Growth curves were also measured for these same cells in the presence of variable numbers of lymphocytes (ratios of 10, 20, 50, and 100 lymphocytes to each melanoma cell). Finally, a growth curve was obtained for melanoma cells in the presence of both lymphocytes and CRP.

Results. Figure 1 summarizes the results of the growth curve studies. Under the experimental conditions described, the melanoma cells doubled in number at an average of 66 hr (64–68 hr). Added CRP (20 µg/ml) did not modify the growth curve. This CRP concentration represented a ratio of 16 µg of CRP to 10^6 lymphocytes and was selected because higher concentrations of CRP (20 µg of CRP/10^6 lymphocytes) had been found to be toxic to lymphocytes (3). Lymphocytes alone, even at a ratio of 100 lymphocytes to 1 tumor cell did not destroy the tumor cells, but the higher numbers of lymphocytes (50:1, 100:1) caused an increase in the doubling time (75 hr as opposed to 66 hr), chiefly by increasing the early lag phase. Once the initial effect was overcome, the melanoma cells went on to multiply normally; and, at 96 hr, there was no significant difference in numbers of melanoma cells between the controls and those exposed to lymphocytes. In both groups, the cells appeared healthy and maintained a 95% viability as determined by vital staining. The test model selected was a 50:1 ratio of lymphocytes to melanoma cells in order to provide an adequate number of functional lymphocytes and yet avoid nutrient depletion by overcrowd-

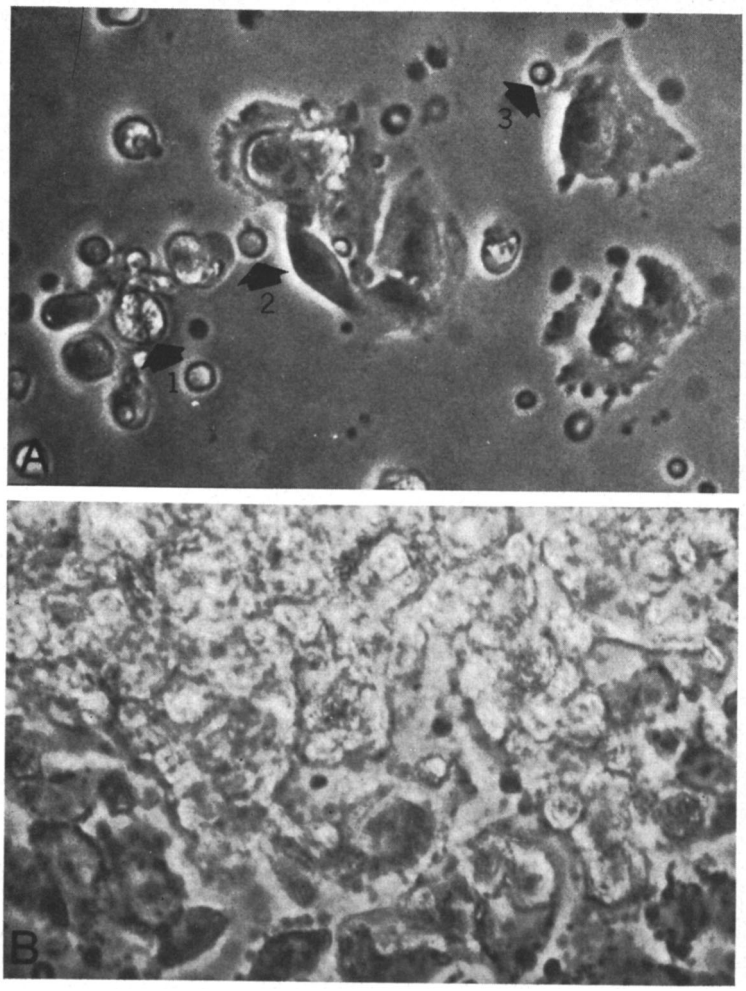

FIG. 2A. Destruction of melanoma cells by lymphocytes in the presence of CRP: (1) extruded nucleus of melanoma cells; (2) lymphocyte with protruding foot process; (3) lymphocyte attached to melanoma cell. Center clump of melanoma cells has attached lymphocytes, and melanoma cell in lower right is breaking up. (B) Growth of melanoma cells in presence of same lymphocytes as in (A) but without CRP: Photographs taken at 48 hr, ×450 magnification. Lymphocyte/tumor cell ratio is 50:1 in both cases.

ing.

When melanoma cells and lymphocytes were cultured together in the presence of CRP, there was no evidence of melanoma cell multiplication. Rather there was a gradual decrease in cell numbers up to 48 hr, at which time the melanoma cells became detached from the wall in large numbers so that, at 72 hr, less than one half of the original inoculum was left attached to the wall and, of these, approximately half were not viable.

Figure 2A shows melanoma cells being destroyed by lymphocytes in the presence of CRP. Arrows point to (i) extruded melanoma cell nucleus; (ii) lymphocyte with protruding foot; and (iii) lymphocyte attached to melanoma cell. Another melanoma cell is in the process of breaking up, and a central small clump of tumor cells has numerous attached lymphocytes. In contrast, Fig. 2B shows a sample of the same melanoma cells and lymphocytes without added CRP. The tumor cells grew actively in the presence of

the lymphocytes. The lymphocyte/melanoma cell ratio (50:1) was the same in both flasks. Both photographs were taken at 48 hr of culture and at the same magnification.

Discussion. Lymphocytes at a ratio of 50:1 changed the growth curve of melanoma cells so that the doubling time was increased from 66 to 75 hr. This was not due to competition for nutrients since the medium was ultimately capable of sustaining the growth of the same number of cells as the controls. Donors of all lymphocytes tested differed from the melanoma cell donor by two HL-A antigens. Therefore, the increased doubling time may represent the effect of histocompatibility differences; and this point will be investigated. In any event, the melanoma cells were not significantly affected by the presence of allogeneic lymphocytes at this concentration. However, when these same lymphocytes were stimulated by CRP (16 μg of CRP/10^6 lymphocytes), the growth of the melanoma cells was completely inhibited; and, in 72 hr, about 75% of them were dead. Lower concentrations of CRP had less predictable effects and higher concentrations were toxic to the lymphocytes themselves. These results show that CRP specifically stimulated lymphocytes and that such stimulated lymphocytes completely inhibited melanoma cell growth when the ratio of lymphocytes to melanoma cells was 50:1. Although the inhibiting effect may be due to release of a humoral agent, the microscopic observations (Fig. 2) suggested rather that the damage to the melanoma cells was due to direct cellular activity by the stimulated lymphocytes. The above experiments strengthen Wood's hypothesis (1, 2) that CRP may play a role (*i.e.*, lymphocyte activation) in the events leading to immune responses.

Summary. When human melanoma cells were grown in tissue culture with allogeneic lymphocytes at a ratio of 50 lymphocytes to one melanoma cell, the growth curve was altered so that doubling time of melanoma cell count increased by 14% over controls. CRP alone did not alter the growth curve; but when CRP (20 μg/ml) was added to lymphocytes and melanoma cells (50:1), no increase in melanoma cell numbers occurred. Rather, there was a decrease in melanoma cells and loss of viability so that, at 72 hr, 75% of the original inoculum was dead. It was concluded that CRP stimulated normal lymphocytes to destroy melanoma cells. The suggestion is made that lymphocyte activation is the normal physiological function of CRP.

The author wishes to acknowledge the technical assistance of Mr. E. Benes.

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Received Sept. 1, 1971. P.S.E.B.M., 1972, Vol. 139.