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# Tumor Colony Formation by Friend Virus-infected Cells in Immunosuppressed Mice[1]

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## SUMMARY

We have investigated the role of host immunological factors in the formation of "tumor colonies" in the spleens of unirradiated C57BL/6 × C3Hf/Bi $F_1$ mice 9 days after i.v. injection of spleen cells from Friend virus (FV)-infected C3Hf/Bi donors. Pretreatment of hosts with antithymocyte serum (ATS) increased the number of tumor colonies. Pretreatment with formalinized FV-infected cells had the opposite effect, and ATS diminished the inhibitory effect of preimmunization.

Cell suspensions from 11 individual FV-infected donors were examined. The suspensions differed with respect to their behavior on transplantation into untreated and ATS-pretreated $F_1$ hybrid hosts. With several suspensions, the number of tumor colonies produced was approximately proportional to the number of cells injected; in all of these, ATS increased the slope of the line relating colony number to cell number. With most of the suspensions, tumor colony-forming efficiencies in untreated hosts strikingly decreased with increasing number of cells injected; ATS induced an increase in the number of tumor colonies and rendered the colony-forming response more nearly proportional to cell number. With two suspensions, few or no colonies developed; pretreatment with ATS had no significant effect. When the 11 cell suspensions were considered together, a proportional relation was found between the magnitude of the ATS effect (*i.e.*, colony number in the presence of ATS minus colony number in the absence of ATS) and the colony-forming efficiency in ATS-treated mice. The ATS effect on the average was equivalent to a 2-fold increase in tumor colony-forming efficiency.

We interpret these findings to indicate that two factors interact to determine the number of tumor colonies produced by spleen cells from FV-infected C3H donors in untreated $F_1$ hybrid hosts. One is a property of the FV-infected cell population and includes its frequency of tumor colony-forming units; this factor varies widely among different cell suspensions. The other is a property of the tumor colony-forming units-host interrelationship and includes the vulnerability of tumor colony-forming units to the host immune response elicited by the injected cells; this factor appears to be constant with different cell suspensions.The present results show that the two factors can be dissociated in immunosuppressed hosts.

## INTRODUCTION

The i.v. transplantation of spleen cells from mice infected with FV[3] into unirradiated histocompatible hosts that are genetically resistant to infection by FV results in the production of macroscopic colonies in the spleen. These colonies have been referred to as tumor colonies, and the entities responsible for their formation have been called TCFU (6). Cell suspensions from different FV-infected donors produce different numbers of tumor colonies. The question arises as to whether these differences in colony-forming efficiency are entirely due to different numbers of TCFU in the different cell suspensions or whether immunological factors play a part in determining the different numbers of tumor colonies observed. We approached this problem by examination of tumor colony formation in animals immunosuppressed with ATS.

## MATERIALS AND METHODS

**ATS.** ATS, designated HAMTS, was obtained from the Medical Research Council of Canada and was prepared by Dr. G. Lamoureaux (Institute of Hygiene, Laval-des-Rapides, Quebec, Canada). The antiserum was raised in horses that received 4 s.c. injections of homogenized mouse (C56BL/6) thymuses, incorporated in Freund's complete adjuvant, followed by 4 i.v. injections of living thymocytes. Before use, the ATS was heated at 56° for 1 hr. The effectiveness and the immunosuppressive potency of this batch of ATS has been described elsewhere (4).

**Mice.** C3Hf/BiUt, C57BL/6Ut × C3Hf/BiUt $F_1$, and SIM/Ut mice were bred in the Division of Laboratory Animal Sciences, Medical Sciences Building, University of Toronto. Mice were housed in plastic cages and supplied freely with water and mouse breeder diet obtained from Teklad Mills, Winfield, Iowa.

**Virus.** FV, obtained in 1963 from the American Type Culture Collection, has since been maintained in SIM/Ut mice of genotype *Fv-1$^{nn}$; Fv-2$^{ss}$* (3). Virus stocks consisted of either filtered supernatants of centrifuged homogenates of

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[3] The abbreviations used are: FV, Friend virus; TCFU, tumor colony-forming unit; ATS, antithymocyte serum; KRP, Krebs-Ringer phosphate medium; PBS, phosphate-buffered saline (Delbecco's).

infected spleens or of resuspended pellets obtained from high-speed centrifuged infected plasma. Titers of FV, expressed in focus-forming units/ml (1), were measured in SIM mice. Virus preparations were stored in liquid nitrogen.

**Preparation of Spleen Cell Suspensions.** C3Hf/BiUt female mice were infected with 5000 focus-forming units of FV. Animals were killed and their spleens removed 14 to 21 days after infection. The whole spleen was minced with scissors, and the cells were washed through a stainless steel wire mesh (110/inch) with ice cold KRP medium. The cells were washed twice with the medium and an aliquot of the cell suspension was counted in a hemacytometer. Cell numbers given represent total counts of nucleated cells. Appropriately diluted spleen cell suspensions (0.5 ml) were injected into the tail veins of $F_1$ hybrid mice.

**Assay for TCFU.** The method of Thomson and Axelrad (6) was used. Nine days after injection of the cell suspensions, spleens were removed and fixed in Bouin's fluid, and macroscopic colonies on the spleens were counted. Results are presented as mean number of tumor colonies ± S.E. For values above 40, standard errors are not included.

**Pretreatment with Formalinized Cells.** A pool of 3 FV-infected C3Hf/BiUt spleen cells was suspended in KRP. A suspension (total volume, 12 ml) containing $7.2 \times 10^9$ cells and 1.4 ml 10% phosphate-buffered neutral formalin was left overnight at 4° on a magnetic stirrer. Thereafter, the suspension was diluted with an equal volume of KRP and the mice were given 0.4-ml i.p. injections ($1.2 \times 10^8$ cells/mouse).

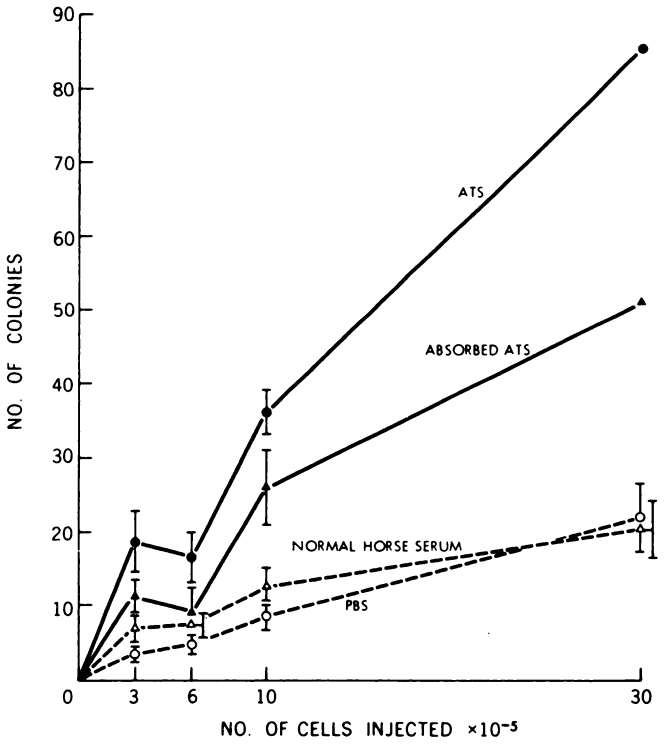


Chart 1. Effect of pretreatment with ATS on tumor colony formation in the spleens of C57BL16 × C3HfBi $F_1$ hybrid mice 9 days after i.v. injection of cells from the enlarged spleen of a FV-infected C3Hf/Bi mouse. ATS (HAMTS) was raised in horses by immunization with homogenized C57BL/6 thymuses. Absorbed ATS was prepared by a 1-hr incubation of 1 ml ATS (HAMTS) with $10^8$ C57BL/6 spleen cells 3 times at room temperature followed by similar incubations with $10^8$ C57BL/6 thymus cells.

## RESULTS

To investigate the effect of ATS on the production of tumor colonies in the spleen, C57BL/6Ut × C3Hf/BiUt $F_1$ mice were divided into 4 groups: Group 1, PBS; Group 2, horse serum; Group 3, ATS; Group 4, the same ATS absorbed with spleen and thymus cells. All injections were given s.c. in a volume of 0.25 ml daily for 7 days. On the 8th day, these hosts were given i.v. injections of different numbers of cells from the enlarged spleens of FV-infected C3Hf/BiUt mice, and the number of macroscopic colonies on the surface of the spleens was scored 9 days later. The results in Chart 1 show that the number of tumor colonies in the spleens of the untreated $F_1$ hybrid mice increased with increasing numbers of FV-infected C3Hf/BiUt cells injected, in agreement with earlier findings (6). Pretreatment of the mice with ATS resulted in a substantial increase in the number of tumor colonies that developed. Normal horse serum had no effect. Absorption of ATS with lymphoid cells significantly reduced the effectiveness of the ATS.

ATS given to susceptible mice given injections of FV is known to increase the number of spleen foci (5). Since virus-induced spleen foci are not reliably distinguishable from tumor colonies in the spleen, it was possible that the increased number of scored tumor colonies was actually due to spleen foci induced by virus carried in with the injected spleen cells. This was tested by a comparison of spleen colony formation in hosts given injections of either irradiated or unirradiated FV-infected spleen cell suspensions. A dose of $10^4$ rads was chosen because it was known that this dose reduces the number of TCFU to infinitesimally low levels (6), while reducing the infectivity of the virus by no more than 10% (2). Table 1 shows that any contribution by free virus is negligible.

We next investigated the question as to whether the ATS effect was attributable to suppression of an immune response called forth by the injected cells. Hybrid mice C57BL/6 × C3Hf/Bi $F_1$ were treated with ATS or were given PBS. On the 8th day, mice of both groups were either immunized with a single i.p. injection of formalinized cells from FV-infected C3Hf/Bi mice or were left untreated as controls. Seven days later, mice of all 4 groups were challenged with $3 \times 10^5$ and with $1 \times 10^6$ FV-infected C3Hf/Bi spleen cells (Table 2). Immunization reduced the number of tumor colonies (compare Groups 1 and 3). Pretreatment with ATS reduced the effectiveness of this immunzation (compare Groups 3 and 4). It is clear that the ATS effect is connected with the immunogenicity of the infected cell suspensions and is attributable to its immunosuppressive action.

It was already evident (Chart 1; Tables 1 and 2; and Ref. 6) that there were wide differences in the tumor colony-forming efficiencies of spleen cell suspensions from different FV-infected donors in untreated $F_1$ hybrid hosts. To obtain information on the contribution of the immune system to these variations, we examined the effect of ATS on tumor colony formation by 11 independently derived FV-infected C3Hf/Bi spleen cell suspensions.

Table 1

*Effect of donor cell irradiation on tumor colony formation*

Animals were given injections with either $3 \times 10^6$ spleen cells or $3 \times 10^6$ irradiated spleen cells from leukemic animals.

| Experiment | Treatment of donor cells | No. of colonies/spleen pretreatment of animal | | Comparison (*t* test) |
|---|---|---|---|---|
| | | None | ATS | |
| 1 | None | 20.8 ± 4.6[a] | 55.6 ± 5.8 | *$p < 0.01$*[b] |
| | Irradiated ($10^4$ rads) | 0.0 ± 0.0 | 0.5 ± 0.3 | $0.05 < p < 0.10$ |
| 2 | None | 16.0 ± 3.5 | 50.1 ± 8.8 | *$p < 0.01$* |
| | Irradiated ($10^4$ rads) | 0.4 ± 0.2 | 0.1 ± 0.1 | $0.30 < p < 0.40$ |

[a] Mean ± S.E.
[b] Significant differences between means are *italicized*.

Table 2

*Effect of immunization and of ATS on tumor colony formation*

| Group | Pretreatment: ATS | Pretreatment: Formalinized cells | No. of cells injected | No. of colonies/spleen | Comparison (*t* test): With Group 1 | With Group 2 | With Group 3 | With Group 4 |
|---|---|---|---|---|---|---|---|---|
| 1a | – | – | $3 \times 10^5$ | 1.7 ± 0.7[a] | | *$p > 0.20$*[b] | *$0.02 < p < 0.05$* | $p > 0.40$ |
| 2a | + | – | | 3.4 ± 1.1 | $p > 0.20$ | | *$p < 0.01$* | $0.05 < p < 0.10$ |
| 3a | – | + | | 0.1 ± 0.1 | *$0.02 < p < 0.05$* | *$p < 0.01$* | | $0.05 < p < 0.10$ |
| 4a | + | + | | 1.0 ± 0.4 | $p > 0.40$ | $0.05 < p < 0.10$ | $0.05 < p < 0.10$ | |
| 1b | – | – | $1 \times 10^6$ | 2.5 ± 1.0 | | *$0.02 < p < 0.05$* | *$0.02 < p < 0.05$* | $0.10 < p < 0.20$ |
| 2b | + | – | | 8.0 ± 1.9 | *$0.02 < p < 0.05$* | | *$p < 0.01$* | $0.10 < p < 0.20$ |
| 3b | – | + | | 0.3 ± 0.1 | *$0.02 < p < 0.05$* | *$p < 0.01$* | | *$p < 0.01$* |
| 4b | + | + | | 4.3 ± 0.9 | $0.10 < p < 0.20$ | $0.10 < p < 0.20$ | *$p < 0.01$* | |

[a] Mean ± S.E.
[b] Significant differences between means are *italicized*.

ATS induced an increase in tumor colony numbers with 8 of 11 cell suspensions (Table 3; Chart 2). With 2 others ($T_8$, $T_9$), practically no colonies developed either in the controls or in the ATS-treated animals. With a 3rd ($T_5$), colony-forming efficiency was very low, and ATS had no effect. Another cell population ($T_2$), of equally low colony-forming efficiency, showed an increase after ATS treatment of the host.

Chart 2 and Table 3 show that the relation between the number of tumor colonies and the number of cells injected varied among the different cell suspensions. In most untreated hosts, the number of spleen colonies was not directly proportional to the number of cells injected over the whole range, the colony-forming efficiency decreasing with increasing cell number. ATS appeared to render the colony-forming response more nearly proportional to cell number. Thus, the greatest effects of ATS were seen at the highest cell doses tested. However, ATS never increased the tumor colony number more than a factor of 5 within the countable range.

Whether any relation existed between the effectiveness of ATS and the colony-forming efficiencies of the different suspensions was next investigated. From the data of the previous experiment, the mean difference in colony number with and without ATS at different cell doses was calculated for each cell suspension and this was plotted against the actual colony-forming efficiency of the same suspension in ATS-pretreated hosts. Chart 3 shows that there was a constant relation between the magnitude of the ATS effect and the colony-forming efficiencies of all the cell suspensions examined; ATS on the average induced a 2-fold increase in tumor colony-forming efficiency.

## DISCUSSION

Pretreatment of C3H × C57BL/6 $F_1$ hybrid hosts with ATS resulted in an increase in the number of tumor colonies after the injection of FV-infected C3Hf/Bi spleen cell suspensions. The number of colonies produced in untreated and ATS-treated mice differed among the different cell suspensions, and for any given cell suspension varied with cell number. When the mean difference in colony number with and without ATS pretreatment was plotted against the mean colony-forming efficiency for the different suspensions, a linear relationship was found (Chart 3). That is, in general, the percentage of increase in colony number due to ATS was independent of colony-forming efficiency. This demonstrates a remarkable degree of uniformity in the effect of ATS on the transplatation behavior of individual FV-infected cell populations, despite wide differences in their tumor colony-forming efficiencies.

Table 3
*Effect of ATS on tumor colony formation*

| No. of cells injected | Cell suspension | No. of colonies/spleen pretreatment of animal: None | ATS | Comparison (*t* test) |
|---|---|---|---|---|
| $3 \times 10^5$ | $T_1$ | 9.9 ± 1.7[a] (7)[b] | 16.9 ± 1.9 (8) | *0.01–0.02*[c] |
| | $T_2$ | 2.0 ± 0.8 (8) | 3.3 ± 0.8 (8) | 0.20–0.30 |
| | $T_3$ | 23.5 ± 5.9 (6) | 36.5 ± 5.3 (6) | 0.10–0.20 |
| | $T_4$ | 9.0 ± 2.3 (6) | 13.3 ± 2.0 (6) | 0.10–0.20 |
| | $T_5$ | 1.9 ± 1.1 (7) | 0.9 ± 0.4 (7) | 0.30–0.40 |
| | $T_6$ | 5.4 ± 1.2 (7) | 8.7 ± 2.2 (7) | 0.20–0.30 |
| | $T_7$ | 6.1 ± 1.9 (8) | 10.4 ± 1.3 (8) | 0.05–0.10 |
| | $T_8$ | 0.0 ± 0.0 (8) | 0.0 ± 0.0 (8) | 0.30–0.40 |
| | $T_9$ | 0.0 ± 0.0 (11) | 0.0 ± 0.0 (11) | 0.30–0.40 |
| | $T_{10}$ | 2.3 ± 0.5 (8) | 4.4 ± 1.5 (8) | 0.10–0.20 |
| | $T_{11}$ | 1.8 ± 0.6 (8) | 4.9 ± 1.6 (8) | 0.05–0.10 |
| $6 \times 10^5$ | $T_1$ | 14.5 ± 2.4 (11) | 10.3 ± 2.8 (6) | 0.20–0.30 |
| | $T_2$ | 1.6 ± 0.5 (8) | 4.9 ± 1.0 (8) | *0.01–0.02* |
| | $T_3$ | 17.2 ± 3.4 (6) | 45.3 ± 8.5 (7) | *0.01–0.02* |
| | $T_4$ | 18.2 ± 4.7 (6) | 18.7 ± 3.1 (6) | >0.90 |
| | $T_5$ | 1.0 ± 0.4 (7) | 1.1 ± 0.5 (8) | >0.80 |
| | $T_6$ | 10.8 ± 1.9 (8) | 20.0 ± 3.0 (8) | *0.02–0.05* |
| | $T_7$ | 11.4 ± 2.1 (8) | 32.4 ± 3.4 (8) | *<0.01* |
| | $T_8$ | 0.0 ± 0.0 (8) | 0.0 ± 0.0 (8) | 0.30–0.40 |
| | $T_9$ | 0.0 ± 0.0 (11) | 0.0 ± 0.0 (11) | 0.30–0.40 |
| | $T_{10}$ | 4.4 ± 1.3 (8) | 10.9 ± 2.2 (8) | *0.02–0.05* |
| | $T_{11}$ | 4.9 ± 1.1 (8) | 22.5 ± 5.9 (8) | *0.01–0.02* |
| $1 \times 10^6$ | $T_1$ | 23.3 ± 2.7 (10) | 44.3 ± 3.2 (8) | *<0.01* |
| | $T_2$ | 2.4 ± 1.1 (10) | 11.6 ± 1.3 (8) | *<0.01* |
| | $T_3$ | 32.6 ± 5.9 (7) | 61.1 ± 6.0 (7) | *<0.01* |
| | $T_4$ | 15.8 ± 3.5 (6) | 38.1 ± 2.8 (7) | *<0.01* |
| | $T_5$ | 2.9 ± 1.0 (8) | 2.5 ± 0.7 (8) | 0.70–0.80 |
| | $T_6$ | 15.3 ± 2.4 (8) | 37.3 ± 3.2 (8) | *<0.01* |
| | $T_7$ | 23.1 ± 3.4 (8) | 33.5 ± 3.4 (8) | *0.02–0.05* |
| | $T_8$ | 0.1 ± 0.1 (8) | 0.3 ± 0.2 (8) | 0.40–0.70 |
| | $T_9$ | 0.0 ± 0.0 (11) | 0.0 ± 0.0 (11) | 0.30–0.40 |
| | $T_{10}$ | 8.9 ± 2.3 (7) | 13.1 ± 2.7 (8) | 0.10–0.20 |
| | $T_{11}$ | 8.4 ± 2.1 (8) | 20.8 ± 4.5 (8) | *<0.01* |
| $3 \times 10^6$ | $T_5$ | 11.3 ± 5.6 (7) | 4.8 ± 0.5 (8) | 0.20–0.30 |
| | $T_6$ | 43.9 ± 6.0 (7) | 90.6 ± 7.2 (8) | *<0.01* |
| | $T_7$ | 33.9 ± 3.1 (8) | 85.8 ± 4.0 (8) | *<0.01* |
| | $T_8$ | 0.5 ± 0.3 (8) | 0.0 ± 0.0 (8) | 0.10–0.20 |
| | $T_9$ | 0.0 ± 0.0 (11) | 0.0 ± 0.0 (11) | 0.30–0.40 |
| | $T_{10}$ | 20.8 ± 4.6 (8) | 55.6 ± 5.8 (8) | *<0.01* |
| | $T_{11}$ | 16.0 ± 3.5 (8) | 50.1 ± 8.8 (8) | *<0.01* |

[a] Mean ± S.E.
[b] Numbers in parentheses, the number of animals in assay.
[c] Significant differences between means are *italicized*.

When individual dose-response curves were examined in untreated and ATS-pretreated animals, it was found that the ratio of number of tumor colonies to number of injected cells decreased with increasing cell number in untreated hosts but was approximately constant in ATS-pretreated hosts. Thus, the injected cells may be considered to have elicited an immune response in the untreated hosts. This would eliminate a proportion of the total population of tumor colony-forming units that could be expressed in immunosuppressed hosts.

The proportion of surviving TCFU in untreated hosts reached a minimum value that was never less than 20 to 50% of that in ATS-treated hosts. This is not due to an inherent resistance to the immune response of a major portion of the TCFU population, since the surviving fraction of TCFU could be reduced to less than 5% by preimmunization (Table 2). The limitation in the magnitude of the immune response against TCFU that can be elicited in untreated hosts may be a consequence of the limited time available before the tumor colonies reach scorable macroscopic size.

Three suspensions, $T_5$, $T_8$, and $T_9$, seemed to behave differently from the rest in that ATS had no significant effect. In all of these, however, the tumor colony-forming efficiencies were very low. Low tumor colony numbers could thus arise either because of an inherently low colony-forming efficiency of the individual cell suspensions, or

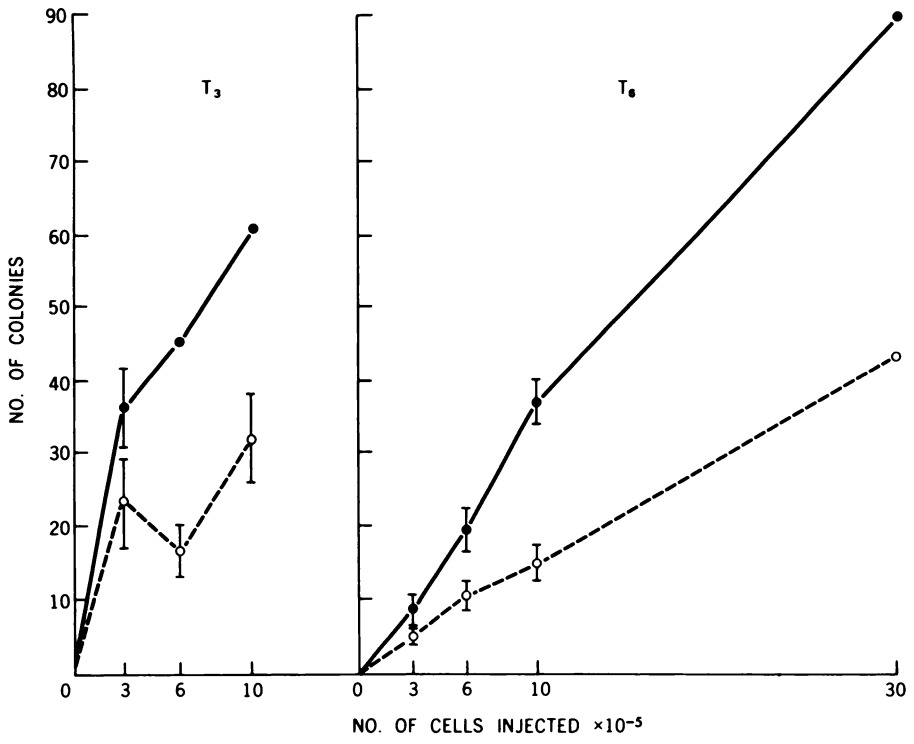


Chart 2. Relation between number of tumor colonies and number of cells injected in PBS- and ATS-pretreated animals. Results obtained with cell suspensions from 2 different FV-infected mice are shown.

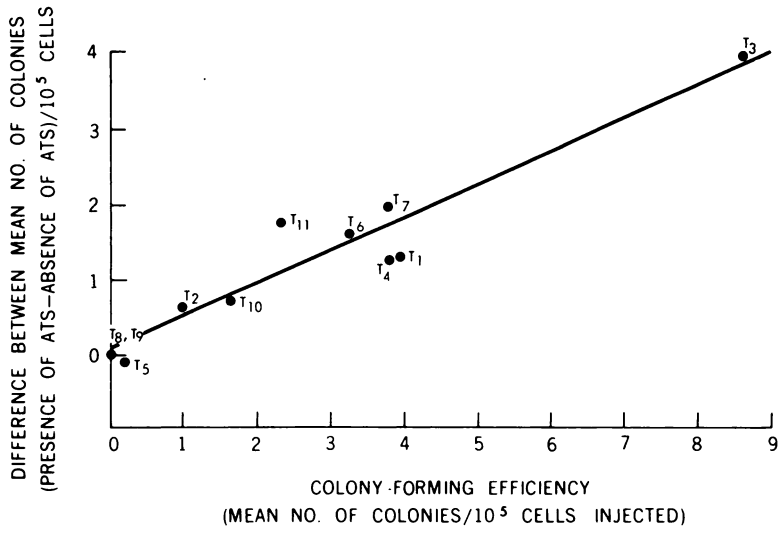


Chart 3. Relation between ATS effect and tumor colony-forming efficiency for 11 cell suspensions from individual FV-infected donors. ATS effect is defined as the difference between mean number of colonies/$10^5$ cells in the presence and in the absence of ATS. For each suspension, this difference was determined at 3 cell dose levels, expressed per $10^5$ injected cells, and the mean was plotted on the *vertical axis*. Colony-forming efficiency is defined as the number of tumor colonies per $10^5$ injected cells in ATS-pretreated animals. For each suspension, this value was determined at 3 cell dose levels and the mean was plotted on the *horizontal axis*.

because of an inherently higher colony-forming efficiency coupled with a significant host immune response against the TCFU (compare $T_5$ with $T_2$).

Thus, the TCFU generated in FV-infected C3H mice are vulnerable to the immune response in C3H × C57BL/6 $F_1$ hybrid hosts. This conclusion is consistent with the earlier serological demonstration of the existence of FV-induced antigen(s) on the TCFU (6). This work indicates that the magnitude of this immunological effect per TCFU is constant and in general amounts to a reduction in colony-forming efficiency by a factor of about 2. In short, we conclude that the overall value for tumor colony-forming efficiency in this system is a variable property of different FV-infected cell suspensions and that this value is lower than the true value as a consequence of the immune response against TCFU.

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