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DISCLAIMER

PRIMARY AVIAN TENDON CELLS IN CULTURE--AN IMPROVED SYSTEM FOR UNDERSTANDING MALIGNANT TRANSFORMATION

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Abstract

Primary avian tendon (PAT) cells which maintain their differentiated state in culture are rapidly transformed by Rous sarcoma virus. By criteria of morphology, increased rate of 2-deoxyglucose uptake, and loss of density dependent growth control, PAT cells transform as well as or better than their less differentiated counterpart, chick embryo fibroblasts. Furthermore, the percentage of collagen produced by PAT cells drops on transformation by an order of magnitude, from 23 to 2.5%, but is unaffected by viral replication of a transformation-defective mutant.

The responsiveness of normal and transformed PAT cells to various environmental factors, changes dramatically upon transformation. Normal PAT cells respond to the presence of ascorbate and high cell density by raising the level of collagen synthesis from 5 to 23%. Transformed PAT cells are totally unresponsive. These and previously reported results, lead us to a "drift" theory of transformation.

INTRODUCTION

Chick fibroblasts, derived from the body wall of chick embryos (chick embryo fibroblasts, CEF) and their Rous transformed counterparts have been used as models for normal and malignant states for many years. There can be no doubt that this system has brought advances in our understanding of viral-transformation; nevertheless, a significant improvement could be obtained by using highly differentiated fibroblasts, which possess tissue-specific functions. A basic assumption in using mixed fibroblast cultures from many tissues is that they all behave the same. While no one would expect epithelial cells from liver, breast, and kidney to behave the same, fibroblasts from the above tissues are treated as if they are all equivalent. Using mixed fibroblast cultures has the added disadvantage of reducing one's ability to define with any specificity the differentiated state of the cells since no clear in vivo reference point exists. Furthermore, cells are grown in complex medium without regard as to whether or not there are detrimental factors present or beneficial factors absent which are necessary for maintaining the differentiated state (21,22). Using a mixed fibroblast population in a medium which is most likely detrimental to the expression of differentiated function, hinders our ability to resolve differences between the normal differentiated state and the transformed state of the cell (19). This may be extremely important if the mechanisms used by the cell to maintain its differentiated state are the ones blocked by the virus to create the transformed phenotype.

To answer the objections above, we have made use of primary avian tendon (PAT) cells (21,22). These cells have several distinct advantages over CEF cells. First, they are derived from a tissue which is composed almost exclusively of a single type of fibroblast. Therefore, we are dealing

with a homogeneous population. Second, PAT cells in the right environment will maintain their differentiated state in culture (22). In vivo, or in culture, PAT cells devote 25-30% of their total protein to collagen (7,22) (the percentage increases by 1-1/2 times when one takes into account that collagen is first synthesized as a procollagen precursor [5]). This extremely biased synthesis in the direction of a differentiated function makes for an easy assay of changes that occur after transformation.

In the present communication, we have two aims: One, to show that highly differentiated PAT cells could be transformed and studied in the same way as less differentiated cell types. By using standard criteria of morphology, loss of density dependent growth control, increase in 2-deoxyglucose uptake and decrease in collagen synthesis, PAT cells can be transformed as well as chick embryo fibroblasts. Two, to study the similarity and differences in the normal and transformed PAT cells' response to environmental factors which affect the stability of the differentiated state (22). An abstract of the work has appeared (23).

MATERIALS AND METHODS

Cell Culture. PAT cells were isolated from a modification (21,22) of the Dehm and Prockop procedure (7). PAT cells (8×10^5 cells in 25 cm^2 flasks; Falcon, Oxnard, CA) were allowed to attach in 5 mls of F12 medium (11) for 40 min. The medium was then changed and the cells were grown in F12 with 0.2% fetal calf serum (Gibco, Grand Island, NY; deactivated 1/2 hr at 56°C) with subsequent daily changes of medium. When ascorbic acid was used in the medium (50 µg/ml), it was added daily from 100x stock. The stock solution of ascorbic acid was freshly prepared every other day.

Virus Infection and Focus Assay. After PAT cells had attached, the medium was changed to 5 mls of F12 with 0.2% serum plus Rous sarcoma virus cloned from single focus. The cells were incubated for 1 hr and then an additional 5 mls of medium was added. The ratio of virus to cell varied from 1:1 to 1:20 as specified in the figure legends. The rate at which infection spread appeared to be more a function of the strain of the virus than the size of the initial innoculum. Focus assays were performed as described previously (3).

2-deoxyglucose Uptake and Reverse Transcriptase Activity. These methods have been described elsewhere (4,24).

Collagen Assay. The cells were labeled with ^3H proline for 3 hr and were assayed using a purified collagenase as described (21,15).

Cell Counts. Cells were removed from the flask with trypsin (0.05%) and gently pipetted to eliminate clumps. They were then counted in a Coulter counter.

RESULTS

Morphology. One of the most dramatic effects of transformation of mixed chick embryo fibroblasts by Rous sarcoma virus is a change in the morphology of the cells: cells round up, pile up, and are more refractile under a phase microscope (3). With PAT cells the effect of transformation is even more pronounced. Normal PAT cells in medium which promotes the differentiated state, low serum (0.2%) and ascorbic acid (50 µg/ml), assume a very round and flat morphology at high density. Under the phase microscope this gives the appearance of a continuous sheet of cells (although under the electron microscope the distinct separation of cells is clear). PAT cells innoculated with Rous sarcoma virus on the day of isolation show a radical change in the morphology at the end of a week in culture. This is shown in Fig. 1. Transformed PAT cells pile up and are more spindle shaped than their normal counterpart. In addition there is an abundance of rounded cells. By the criteria of morphology PAT cells are transformed.

Growth Control. Another common criteria of transformation is a lack of density dependent growth control. Almost by definition a tumor *in vivo* has to have an impaired sensitivity for cell density, although the rate and the range of cell overgrowth can vary immensely depending on the type of tumor. In culture, transformed cells tend to exhibit what appears to be an exaggerated loss of density dependent growth control. In this regard PAT cells are no exception: after infection by Rous sarcoma virus, PAT cells do not show the normal sharp decline in generation time on reaching a confluent monolayer. The growth curves for normal and virus infected PAT cells (Fig. 2) show that normal cells go from a generation time of about 1 day in the early part of the week, to one which approaches zero growth at the end of the week. The transformed PAT cells are much less inhibited and the generation

time only increases from 1 day to 2 days as the cells reach high density. Also shown in Fig. 2 is the fact that PAT cells infected with a transformation defective virus grow at a rate which is similar to normal cells. This common control indicates that loss of density-dependent growth inhibition is a property of viral transformation and not viral infection and replication. To assure that infection by the defective virus has indeed occurred, we assayed for the presence of virus particles in the medium by looking for the presence of reverse transcriptase (24). On a per cell basis, on day 7, the level of enzyme activity as measured by incorporation of deoxythymidine triphosphate was approximately the same for the wild type and the mutant virus (data not shown).

By exhibiting loss of density dependent growth control, PAT cells appear to be well transformed.

2-deoxyglucose Uptake. The rate of uptake of glucose is a frequently used measure of transformation: transformed cells transport glucose much more readily than their normal counterpart (3). By using the non-metabolizable analog, 2-deoxyglucose, uptake (plus the first step in phosphorylation) can be easily measured by accumulation of the label within the cell. In this respect also, PAT cells respond to being transformed in a typical fashion. The uptake of 2-deoxyglucose per µg of protein of normal and transformed PAT cells is presented in Fig. 3. The rate is 15-fold greater in the transformed cells than in the normal cells.

Collagen Synthesis. While a drop in the level of collagen synthesis is not a widely accepted criterion of transformation, over the past fifteen years several laboratories have shown a positive correlation between transformation and a decline in the percentage of collagen synthesis (10,13,14,17). However, in all these cases, the normal cells synthesized from 6 to 30 fold less collagen than normal PAT cells. With the low level of differentiated

synthesis, the question has been raised as to whether or not the changes observed on transformation reflect the actual process in vivo (17). With PAT cells, we can test the action of Rous sarcoma virus on a cell which approximates the in vivo situation much more closely (22).

To study the action of the transformation on the ability of PAT cells to synthesize collagen, cells were infected with Rous sarcoma virus and the level of collagen synthesis was measured over a one week period. This was compared to normal cells and cells infected with a transformation--defective virus. Under this protocol little change was expected until 4-5 days when infection has spread to a majority of the culture. We therefore concentrated our analysis to the latter part of the week. The data is presented in Fig. 4. By the fifth day, as the cells began to show alteration in the morphology, collagen synthesis began to drop. Over the next two days, transformation proceeded swiftly and collagen synthesis declined steadily. In this experiment, by the end of the week there was a drop in collagen synthesis on transformation from 23% to 7.5% (other experiments, where complete transformation of the culture was achieved within the week, showed a greater decline to 2.5% [Fig. 5]). This drop was a function of transformation and not just virus replication since cells infected wtih a transformation-defective virus synthesized the normal percentage of collagen. From this experiment, we can conclude that transformation has a decisive effect on collagen synthesis of PAT cells which agrees with most of the observations reported in the literature with other fibroblasts (10, 13,14,17).

Responsiveness to External Factors. While a decline in collagen synthesis parallels the degree of transformation in PAT cells, their interrelationship is unclear from the above experiment. We would like to distinguish between two possibilities. One, that the control of collagen synthesis after transformation is still normal but at a reduced level. Two, that

normal control of collagen synthesis has been disrupted and what remains is a residual synthesis which does not respond to "normal" control mechanisms. These two mechanisms are basically distinguished by whether or not a quantitative loss in collagen synthesis reflects a radical change in the responsiveness of the cell. Normal tendon cells in vivo or in culture are sensitive to their environment and only synthesize a higher percentage of collagen when ascorbate is present and the cells are at high density (22). The question can then be raised as to whether transformed PAT cells are also sensitive to these same factors.

To test this possibility, we looked at the ability of normal and transformed PAT cells to modulate the level of collagen synthesis when ascorbate was added to ascorbate-deficient cultures. Normal PAT cells respond dramatically to ascorbate by increasing their collagen synthesis 3-fold from 8 to 23% (Fig. 5). Transformed PAT cells on the other hand, are insensitive to ascorbate, making approximately 2.5% with or without the addition of the vitamin. Transformed PAT cells are not only insensitive to concentrations of ascorbate and to density dependent inhibition of growth (Fig. 2), but also to cell density stimulation of collagen synthesis. As has been shown before (21), and is displayed again in Fig. 5, normal PAT cells seeded at a low cell density respond to a small degree to a density increase even in the absence of vitamin C. If one compares normal and transformed PAT cells which were not given vitamin C, then one sees that as the cells reach high cell density at the end of a week in culture, the normal cells respond and raise their synthesis of collagen slightly from 4.5 to 8%; the transformed cultures remain unresponsive within experimental error. Thus, the difference between the sensitivity of the normal PAT cell to its environment and the lack of sensitivity of its transformed counterpart is clearly established.

DISCUSSION

In this paper we have shown that a highly differentiated fibroblast culture can be transformed by RNA-tumor viruses as easily as its less differentiated predecessors. By several of the accepted criteria of transformation: altered morphology, increased rate of 2-deoxyglucose uptake, and loss of density dependent growth control--PAT cells can be transformed by oncogenic viruses. Because the gap between the "normal" differentiated state and the transformed state is broader in PAT cells than in CEF cultures, several of the virus-induced changes have been magnified and are thus easier to study.

In addition we have shown that the synthesis of collagen, the major differentiated product of PAT cells, is impaired after transformation by over an order of magnitude. This result appears to agree with a number of previous studies which have shown several fold drops in collagen synthesis upon transformation by oncogenic viruses (1,10,13,14). While in these studies the relative change in the level of collagen synthesis upon transformation was similar to that in PAT cells, the initial level of synthesis of the "normal" cells was either less than, or only slightly higher than a transformed PAT cell. Comparing in several cell systems the ratio of collagen synthesis before and after transformation becomes confusing when such large differences exist in the "normal" cells. Two perspectives can be taken with respect to the large quantitative difference between the "normal" level of collagen synthesis in PAT cells and other cell systems. First is that the ability to respond is critical, while the actual quantitative level achieved is of only minor importance. The second view is that the type of response that a cell has to transformation can be radically different depending on its initial state. In the latter perspective one would argue that a change in collagen synthesis from 23% to 2.5% (for PAT cells) may be by quite a

different mechanism than a drop observed from 2% to 0.5% (for 3T3 [16,17]). While we have no direct evidence that the changes which occur when PAT cells are transformed are different from that of various cell lines, we do know that the response of the respective normal cells is different towards various external factors. For example, in trying to mimic scurvey in cell culture, PAT cells respond to vitamin C by a mechanism which is similar to cells in vivo (2). They lower the percentage of collagen produced (22) and reduce the level of hydroxylation of proline (unpublished results). Cell lines appear only to alter the level of hydroxylation (16). Therefore, changes in the level of synthesis can affect the type of response as well. PAT cells approximate the "normal" differentiated state more accurately and they may also approximate the transformation process more faithfully.

One reason why PAT cells respond more dramatically to the transformation process is that they will grow at a very low serum concentrations (0.2%). High serum concentrations (>1%) cause the same changes as viral transformation only to a lesser degree: collagen synthesis declines, the cells no longer respond to ascorbate or density dependent growth control, and morphology is changed in the direction of virally transformed cells (20,21,22, and unpublished results). Therefore, starting with "normal" cells in high serum could significantly reduce the effect of viral transformation.

Taken together, the effects of serum and Rous sarcoma virus on PAT cells leads to a theory of transformation which is different from the usual perspective. We will refer to the two views of malignant transformation as the "directed" and the "drift" hypotheses. In the "directed" view, transformation occurs because of an active takeover of the cell which bypasses the normal mechanisms used by the cell to maintain the differentiated state. This could be easily imagined as the synthesis of a gene product (src gene or a cellular transformation gene) which overtakes the normal cell and

transforms it. The critical point is that transformation is by a mechanism which is new to the normal cell, and which is not the "normal" response of the cell to foreign stimuli. A bias towards this approach leads one to concentrate on the transformed cell and the properties that it has newly acquired in an attempt to pinpoint the action of the "directed" takeover. In this view the normal cell is relegated to a background position from which one can judge the new changes acquired by the transformed cell. In taking a "directed" perspective, understanding the control mechanisms of the normal cell would be of dubious value since transformation would occur by an alternate route.

In the "drift" hypothesis, the important element is the "differentiated state" of the normal cell. That is the normal cell's need to be constantly responding to its environment in a correct fashion in order to remain normal. Interference with the cell's ability to sense its environment leads to a "drift" away from the differentiated state towards the transformed phenotype. In using the word "drift" we do not mean to connote a slow change or a random change, but only an alteration in the capability of the cell to maintain the "differentiated state". This view would predict the possibility that transformation could have a continuum of abnormal phenotypic states, the end result of which would be a typical neoplastic state. With this perspective, malignant transformation would be open to a wide variety of agents which could interfere with the cell's ability to sense the environment and stay differentiated. Assuming the plausibility of the "drift" model, one would concentrate on studying the normal cell in order to clarify the operative control mechanisms used by the cell to maintain the differentiated state. The virally-transformed cells would then provide the extreme example of loss of control mechanisms.

Of the two alternatives discussed above, the "directed" model underlies most cancer research. This is perhaps understandable since normal control mechanisms are usually unknown but new characteristics appearing after transformation are easy to enumerate. However, this experimental bias towards the "direct" model is unfortunate since strong arguments can be made for a "drift" model of malignancy, especially in the case of spontaneous tumors in animals and human cancers.

One reason for favoring the "drift" model is that the normal cell is unstable in a foreign environment. The clearest example is the transition from in vivo to culture which almost inevitably yields a cell which dedifferentiates (6). Moreover, this "drift" appears to widen as the length of time in culture increases. Even with PAT cells where the in vivo levels of function in culture may be maintained (22), the cells remain extremely sensitive to small changes in their environment. Therefore, at least within a certain range, the natural "drift" occurs whenever the environment changes. What is not clear is whether cells can "drift" and become transformed. While suggestive evidence for this could come from the effect of high serum on PAT cells, where serum causes a transformed phenotype (as described above), better evidence comes from teratoma and teratocarcinoma cells. Teratomas and teratocarinomas (18) can be derived by taking normal cells from early embryos and placing them in other tissues of the adult (8). These tumors that form are not uniform in that they are made up of a disorganized variety of differentiated phenotypes. Most importantly, if these cells are returned to their normal enviornment, another embryo, they will mature normally to form a healthy individual (12,8). Thus the environment determines whether or not the growth of these cells will be a tumor or part of a healthy mouse. This type of cell behavior is difficult to explain by a "directed" activation of a transformation gene but is easily explained by a "drift" model. Normal embryonic cells placed in an abnormal environment "drift" and become transformed.

In this strange setting they take on phenotypes in a disordered manner. When placed in a normal location, they regain their original state. In this case, the cell does not permanently become changed, but always remains responsive to its environment. One may conclude that some varieties of cancer (and the initial event of many others) may exist with normally responsive cells but in an aberant environment.

Perhaps the strongest argument for a "directed" mechanism comes from viral-transformation, especially Rous sarcoma virus infected avian cells. In this case the product of one gene (src) appears to overwhelm and transform the cell. However, when this example is looked at more closely as in this paper with PAT cells, one sees that the cell appears to be prevented from its ability to sense its environment. The transformed cell does not respond to either ascorbate or cell density, both of which have radical effects on the normal cell. Thus, another interpretation of viral transformation could be that it causes a block in the normal control mechanisms of the cell and in so doing sets the cell to "drift", albeit rapidly. Understanding the action of ascorbate and cell density on the normal cell may lead to a finer resolution of the essential control mechanism than would a search to locate the action of the src gene amongst a myriad of secondary changes in the transformed cell.

At this time too little is known about either the normal control mechanisms of the cell or the process of transformation; therefore, it is difficult to argue strongly in favor of the "directed" or the "drift" model. The use of differentiated cultures such as PAT cells allows one to study the critical changes without experimentally induced bias. With PAT cells one can study either the control mechanism used by the normal cell to maintain the differentiated state or the new characteristics acquired after transformation. The inability in previous cell culture systems to study the former events may be one reason why the mechanism by which cells become malignant has remained obscure.

FIGURE LEGENDS

Figure 1: PAT cells grown for one week in F12, 0.2% fetal calf serum and 50 µg/ml ascorbate with daily change of medium. A. Normal cells. B. Cells infected on the day of isolation with Schmidt-Ruppin A subgroup of Rous sarcoma virus. The ratio of virus to cells was 1:20 as determined by focus forming units.

Figure 2: Growth curves for normal cells (○), cells infected with wild type Prague C, Rous sarcoma virus, (△); cells infected with transformation defective mutant of Prague C, Rous sarcoma virus. (◎). Cells were grown in F12, as described in the legend to Fig. 1. The ratio of wild type virus to the cells was 1:1.

Figure 3: The uptake of 2-deoxyglucose in normal (○) and virally transformed (△) PAT cells on day 6 after isolation. The conditions are the same as those described in the legend to Figure 1.

Figure 4: The percentage of collagen synthesis over a one week period for normal cells (○); cells infected with wild type Rous sarcoma virus, (△); and cells infected with a transformation defective mutant (◎). The conditions were the same as described in the legends to Figure 2. The left ordinate expresses the percentage of radioactive proline which was incorporated into collagen relative to total protein. The right ordinate corrects for the fact that proline occurs 5.2 times more often in collagen than in the average protein (9,22). The corrected value is the one referred to in the text.

Figure 5: The effect of addition of ascorbic acid to the medium of normal (○) and virally transformed (△) PAT cells. Normal PAT cells and cells infected with Schmit-Ruppin Rous sarcoma virus (20:1, respectively), were grown for 5 days in medium deficient in ascorbic acid (-). On the 5th day, half the cultures of each set were switched to medium containing vitamin C (--).

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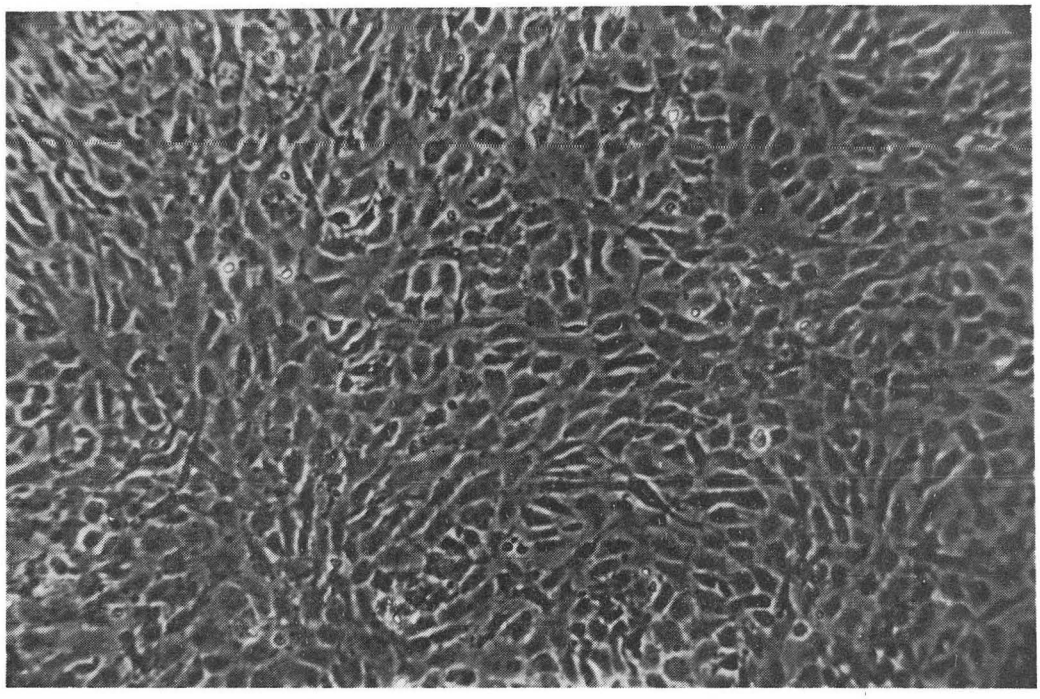
A



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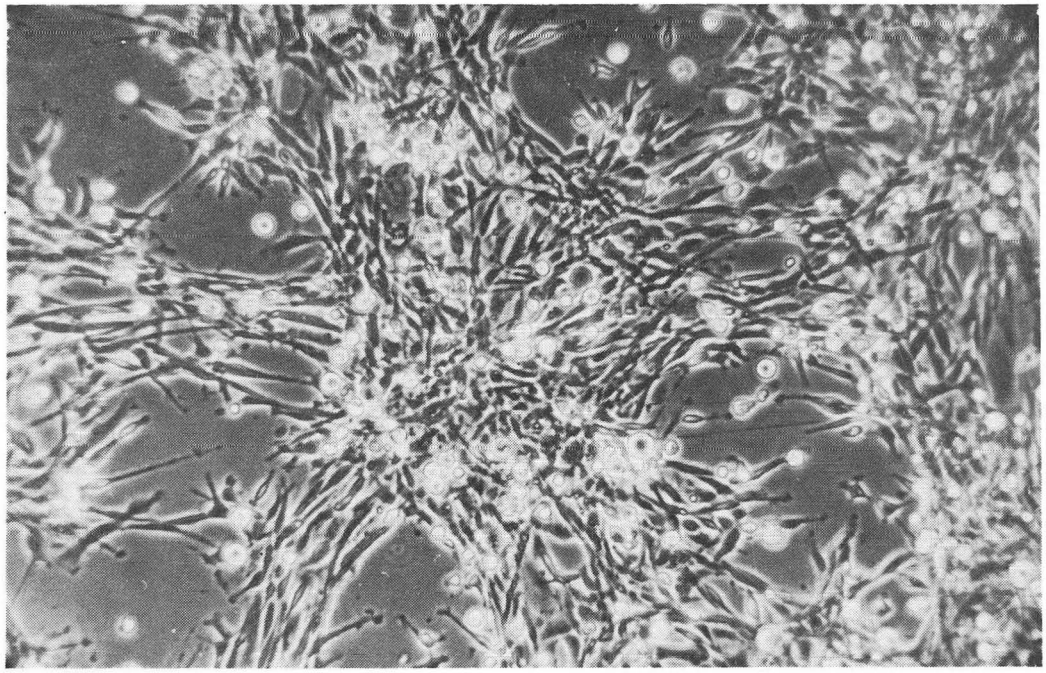

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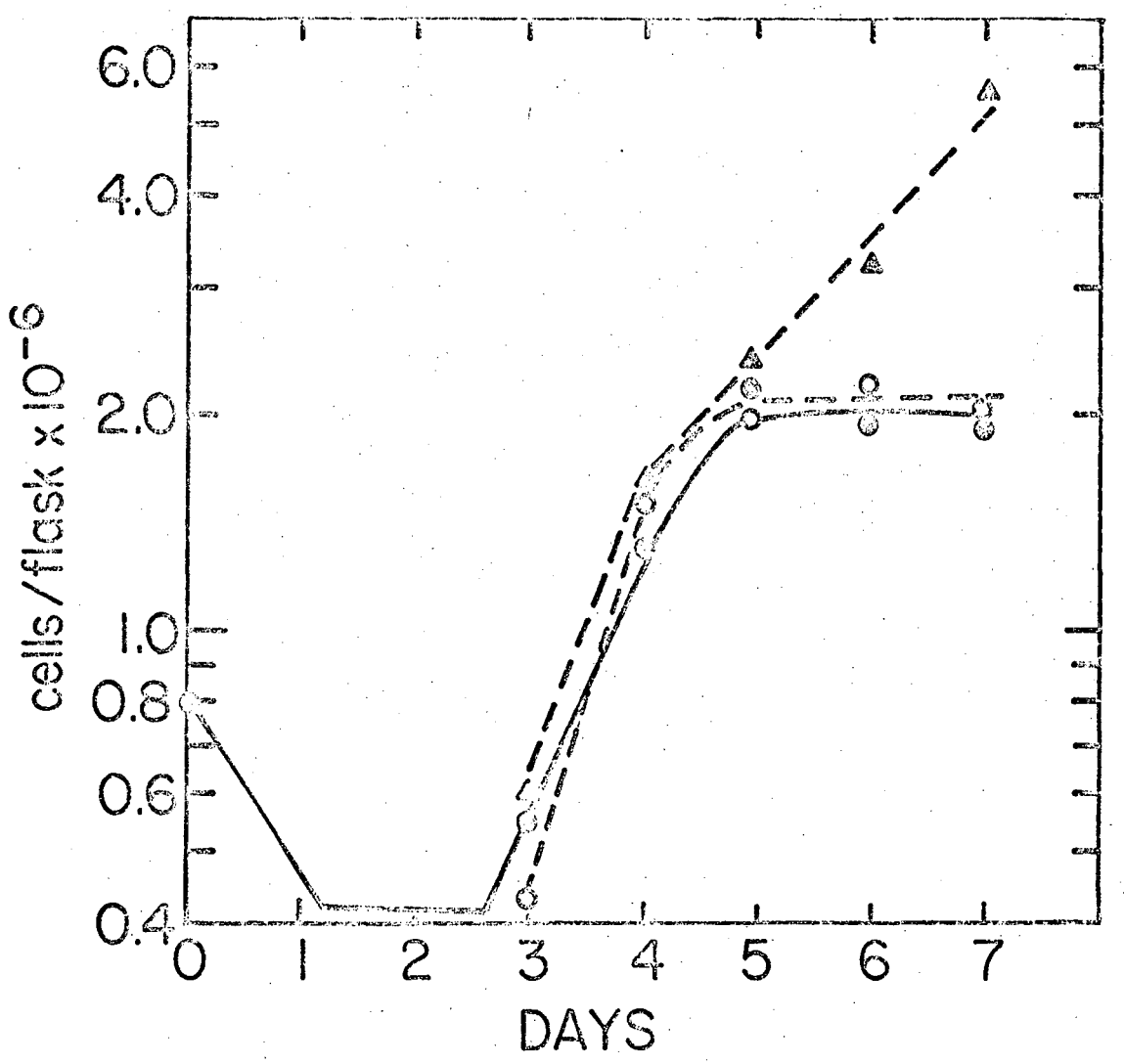
Fig. 1



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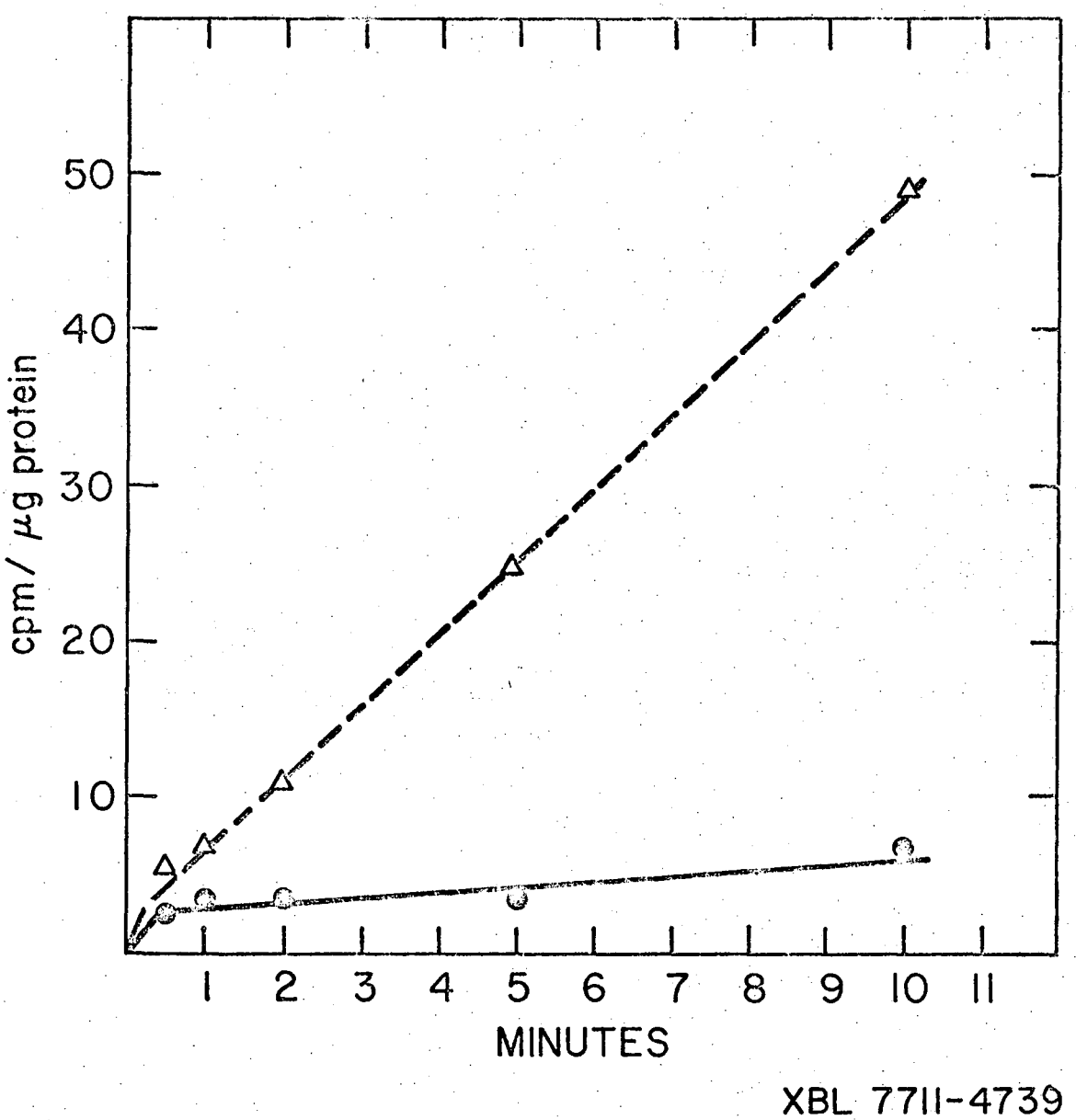
— Figure 2 Schwarz et al. —



— Figure 3 Schwarz et al. —

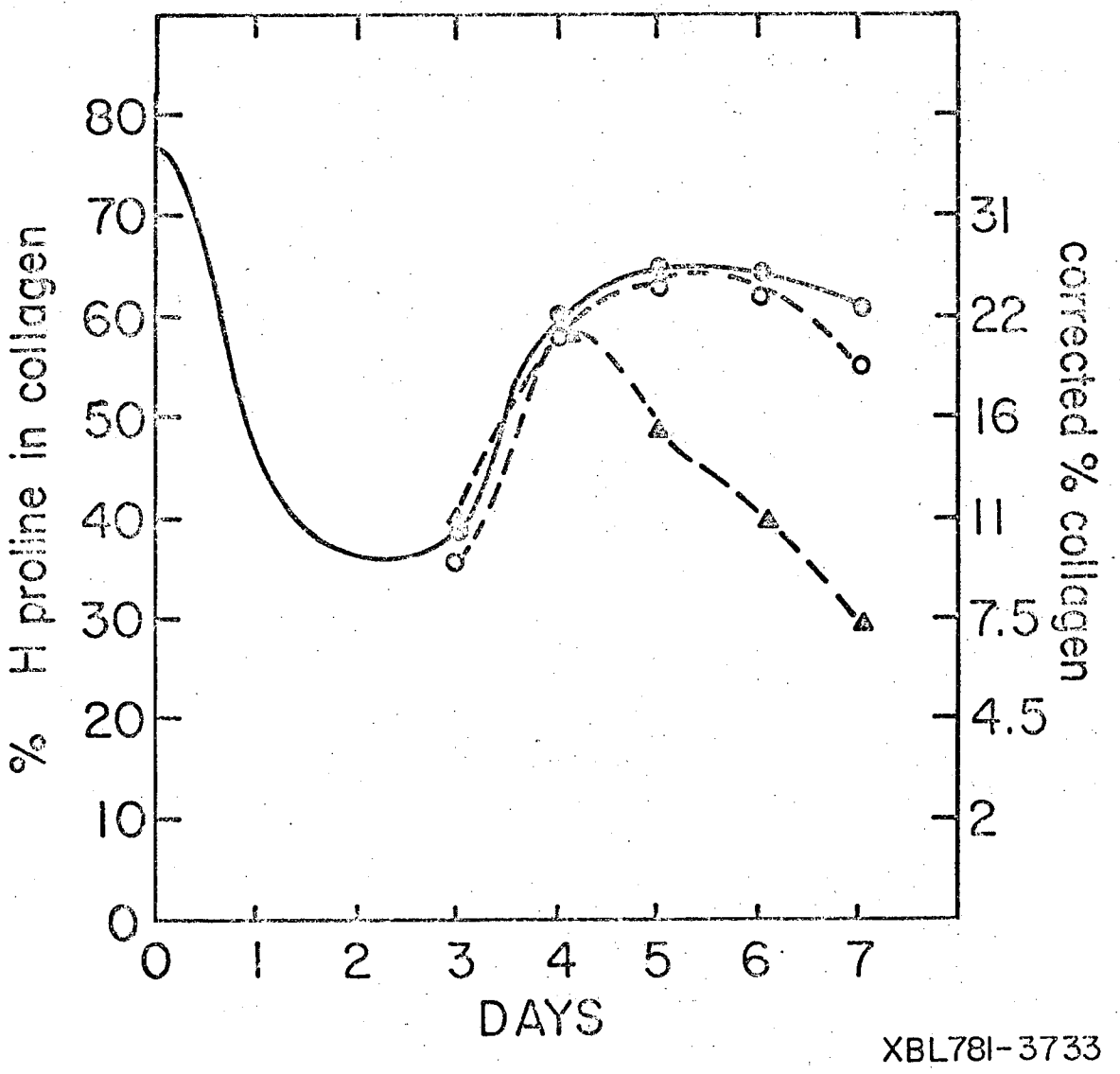


Figure 4 Schwarz et al.

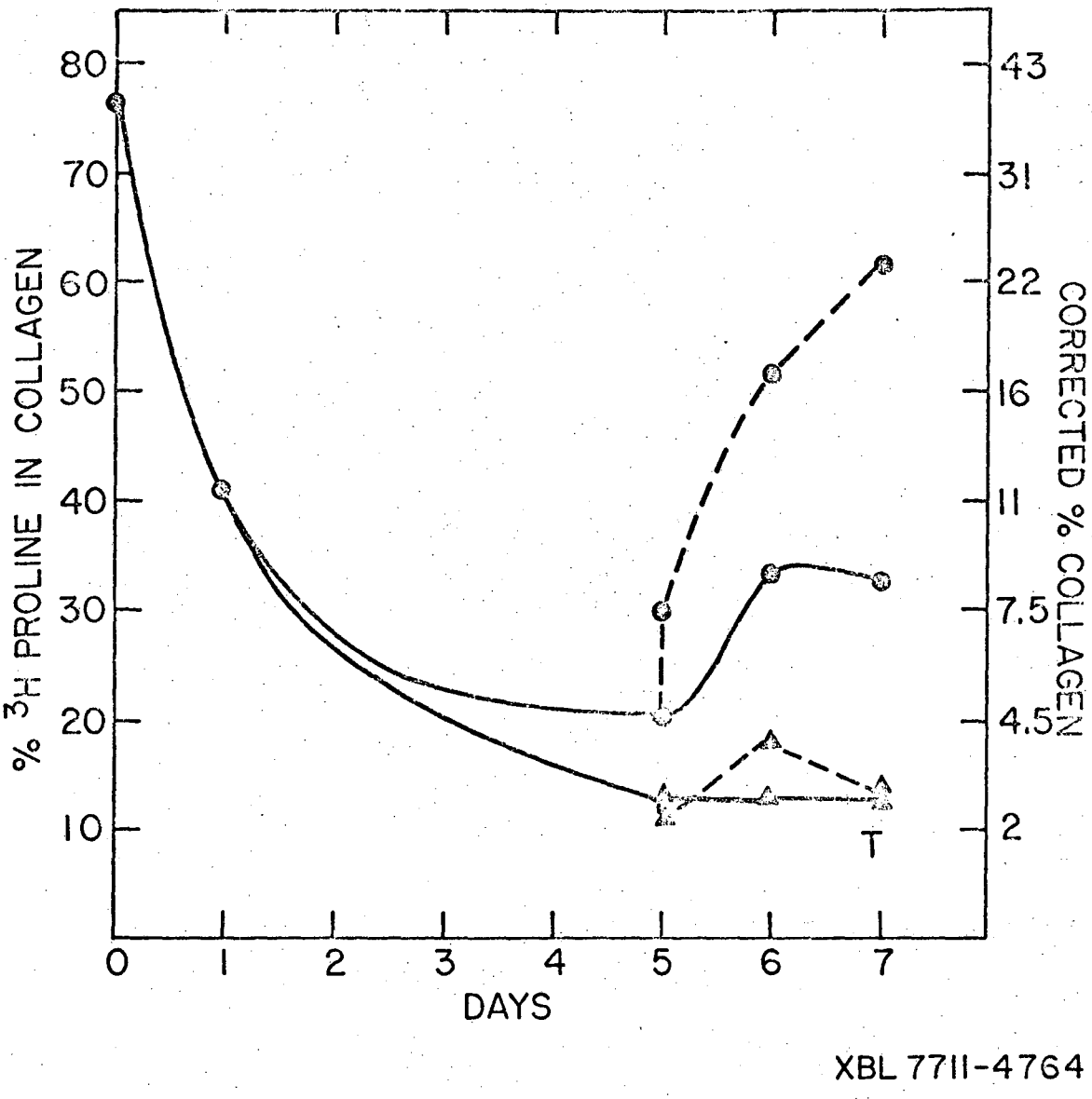


Figure 5 Schwarz et al.

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