Short Communication

Development of insusceptibility to serum factor during the radiation transformation process

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The dependence of transformation, either spontaneously developed or induced by exogenous agents, upon serum has been described by many investigators (Evans & Anderson, 1966; Carbone et al., 1974; Little, 1979) but the reasons for this intriguing phenomenon have not been elucidated. Recently, Terasima et al. (1981) showed that (1) transformation frequency depended not only on the serum used for pre-irradiation culture (culture serum), but also to a considerable extent on the serum used for assay culture (assay serum) when $10^4$ mouse cells in the plateau phase were subjected to X-ray irradiation; and (2) the culture serum affected, in a batch-dependent manner, the removal of transformation damage which occurred during the first 6h of the post-irradiation period. The present studies confirm the effect of assay serum on the transformation frequency, and, by taking advantage of this finding, the time interval required for the development of insusceptibility to the serum factor in radiation-initiated cells was determined.

The cell line used was $10^4$ (clone 8; Reznikoff et al., 1973a) kindly provided by Dr. C. Heidelberger (University of Wisconsin). Experiments were carried out with cells between the 6th and 13th passages. The cultures were grown in Eagle's basal medium supplemented with 10% heat-inactivated foetal calf serum (both obtained from Flow Laboratories, USA; a part of the serum was from the Japan Cancer Research Association Fund operated by Y. Ikawa, Cancer Institute, Tokyo), penicillin ($100 \mu g \cdot ml^{-1}$) and streptomycin ($100 \mu g \cdot ml^{-1}$), unless otherwise stated.

Essentially all the procedures and criteria to determine transformation quantitatively followed those described by Reznikoff et al. (1973b). Pre-irradiation cultures were initiated with $5 \times 10^4$ cells per 60-mm plastic dish and attained confluence on the 6th day, when the medium was renewed. On the 11th day, when the cell density was $3.8 \times 10^4 \cdot cm^{-2}$ ($7.4 \times 10^5$ cells per dish), i.e. the plateau phase, DNA-synthesizing cells represented only 0.19% of the population. The cultures in this state were irradiated without medium renewal. Each culture was trypsinized immediately with 0.1% trypsin solution and the dispersed cells were replated into 100-mm plastic dishes for focus assay at a density such that 300–400 colonies developed in each dish. Each experiment was normally done with a total of 150 dishes; 20–30 dishes were used for each experimental point. Medium renewal was carried out weekly for 8 weeks after irradiation. Fixing and staining of cells and scoring of foci were carried out as described in the preceding paper (Terasima et al., 1981). Tumorigenicity testing of 19 transformed clones revealed that 93% of type III foci after Reznikoff and 25% of type II foci developed tumours in syngeneic C3H/He mice. The results will be reported in detail elsewhere. The transformation frequency was determined from the total number of transformed foci divided by the total number of survivors in assay dishes. The quantitation of transformed foci was carried out on the basis of a Poisson distribution of transformants among assay dishes, since satellite foci rarely developed among assay dishes after an 8-week incubation period, making individual scoring of transformational events impossible (Terasima et al., 1981). Survival was determined in triplicate 60-mm plastic dishes seeded with appropriate numbers of trypsin-dispersed cells. The plating efficiency of plateau phase cells was $\approx 10–15\%$, depending on the batch of serum. X-ray irradiation was delivered from a Shimadzu X-ray generator operated at 200kVp and 20mA with added filtration (HVL: 2mm Cu). Plastic culture dishes placed on a turntable were irradiated with 3.72 Gy at room temperature at a dose rate of 0.5 Gy min$^{-1}$.

Table 1 indicates the batch dependence of transformation among assay sera. The upper 4 determinations were carried out with the same batch of culture serum but different assay sera. However, the transformation frequency was found to be significantly greater with 3 of the assay sera ($\#90509$, $\#81201$, and $\#91011$) than with the...
Table 1  Effect of different assay sera on radiation-induced transformation

| Experimental number | Culture serum (lot no.) | Assay serum (lot no.) | Transformation frequency \((x \times 10^{-4})\) \((\pm\text{s.d.})^*\) |
|---------------------|------------------------|-----------------------|------------------------|
| B-60,61,62          | 81231                  | 81231                 | 8.3 ± 1.6              |
| 65,67-1             |                        |                       |                       |
| B-73,74             | 81231                  | 90509                 | 36.0 ± 4.9             |
| B-67-2              | 81231                  | 81201**               | 48.7 ± 6.2             |
| B-70,71,72          | 81231                  | 91011                 | 24.2 ± 4.7             |
| B-72-2***           | 70324                  | 70324                 | 17.8 ± 7.3             |
| B-72-2***           | 70324                  | 91223**               | 91.6 ± 20.6            |

*The frequency was obtained from the number of induced foci divided by the number of survivors after 3.72 Gy X-ray irradiation.

**New born calf serum.

***Paired experiments.

Figure 1  Development of assay serum independence during transformation assay.

![Figure 1](image)

fourth (#81231). A similar result was found in a paired determination, as shown in the lower 2 lines of the table; the use of a newborn calf serum (#91223) for the assay gave a 5 × greater value of transformation than a foetal calf serum (#70324). We refer to these sera as high yield sera (HYS) and low yield sera (LYS), respectively. In accord with our previous report (Terasima et al., 1981), the present results indicate that different batches of assay serum have significantly different effects on the X-ray-induced transformation yield. In connection with the involvement of serum in chemically-induced transformation, Bertram (1977) indicated that the high serum concentration reduced the yield of de novo transformation, possibly due to an increase in the saturation density of normal cells. However, this explanation does not seem to be applicable to our results, since different batches of serum used at the same concentration did not appreciably change the saturation density of normal cells (data not shown).

The difference in transformation yield with different assay sera could conveniently be used to investigate the temporal change in the susceptibility of cells to serum factor. Pre-irradiation culture prepared with LYS (#70324) was irradiated with 3.72 Gy and immediately subjected to the assay procedure. The assay culture was carried out in 5 groups and involved a sequential shifting from LYS to HYS, as illustrated in Figure 1. The group in which HYS (#91223) was used from the beginning gave a frequency of 91.6 × 10^{-4}, whereas the other groups, in which the shift was made 2 weeks or more later, gave transformation yields comparable.
to that obtained when LYS was used throughout. This result suggests that the culture became insusceptible to HYS within 2 weeks.

Similar experiments were repeated with another pair of LYS (♯16142921) and HYS (♯91223). As shown in Figure 2, the transformation frequency fell quite rapidly at first, and then decreased more slowly, reaching 30% of the initial value after 17 days; it remained essentially constant thereafter. The curve was obtained from pooled data of up to 5 separate experiments, as listed in Table II. The results shown in Figures 1 and 2 demonstrate that radiation-initiated cells were most susceptible to HYS at the beginning of assay culture and thereafter became less susceptible as time passed, up to ~2 weeks. Thus, it appears that initiated cells proceed into a state non-responsive to HYS as cell divisions occur after plating. After 2 weeks of incubation, the transformation yield is comparable to that obtained with LYS alone, indicating that all the initiated cells had become non-responsive to HYS. The half time was ~3 days. This state may be considered to be an intermediate stage toward transformation, since morphological phenotype only becomes apparent after a few weeks of further incubation.

The reverse experiment, where cultures initiated with HYS were subjected to replacement with LYS at various times after irradiation, could not be carried out because insufficient LYS was available.

It is known that initial transformation damage induced by irradiation or chemical carcinogens is fixed as a transformed state within 1 or 2 cell divisions (Borek & Sachs, 1968; Kakunaga, 1974) and several additional generations are needed for full expression, as identified by focus formation (Kakunaga, 1974; 1975). Further investigations have revealed that phenotypic expression of morphological transformation depends on cell-to-cell interactions (Haber et al., 1977; Okada & Watanade, 1978; Bertram, 1979; Kennedy et al., 1980). Based on the above results, one may postulate that initiated (or damage-fixed) cells can be modulated with respect to transformation by a serum factor; the modulated response then becomes

![Figure 2](image-url)  
**Figure 2** Change in response of radiation-initiated cells to assay serum. HYS: high yield serum. LYS: low yield serum. The 3.72 Gy-irradiated cells were grown initially with LYS, and this was replaced by HYS at the times indicated. Transformed foci were scored after 56 days of assay culture. Data are taken from Table II.

### Table II  Transformation frequency as a function of time of serum replacement after plating

| Time of serum shift* after plating for assay | Pooled data | Transformation frequency (× 10⁻⁴) (± s.d.) after 3.72 Gy |
|---------------------------------------------|-------------|----------------------------------------------------------|
| *No. of experiments*                        | *No. of foci/No. of survivors scored* | |
| 0**                                         | 5           | 62.9/32,112                                               | 19.6±2.5                       |
| 1                                           | 3           | 28.1/18,936                                               | 14.8±2.8                       |
| 3                                           | 3           | 23.1/18,341                                               | 12.8±2.6                       |
| 7                                           | 4           | 29.6/24,318                                               | 12.2±2.2                       |
| 10                                          | 2           | 16.7/17,787                                               | 9.4±2.3                        |
| 17                                          | 2           | 11.2/18,906                                               | 5.9±1.8                        |
| 56***                                       | 2           | 10.1/15,698                                               | 6.5±2.0                        |

*The time at which the low yield serum (♯16142921, precolostrum newborn serum, Mitsubishi Kasei Co., Tokyo) was replaced with the high yield serum (♯91223, newborn serum, Flow Laboratories).

**The high yield serum was used throughout the assay period.

***The low yield serum was used throughout the assay period.
fixed, as represented by the development of insusceptibility to HYS, and this is followed by expression of morphological phenotypes via cell-to-cell contact.

From the practical point of view, the present results indicate that it should be possible to use any batch of serum for transformation assay, after the critical period for initiated cells has elapsed.

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