FUSION OF ERYTHROCYTES AND OTHER CELLS WITH RETENTION OF ERYTHROCYTE CYTOPLASM

Nuclear Activation in Chicken

Erythrocyte-Melanoma Heterokaryons

NEHAMA ZAKAI, ABRAHAM LOYTER, and RICHARD G. KULKA. From the Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

INTRODUCTION

Harris and his associates showed that when adult chicken erythrocytes were fused with actively dividing cells, the chicken erythrocyte nuclei in the resulting heterokaryons were activated and began to synthesize both DNA and RNA (1). In these experiments only the nucleus of the chicken erythrocyte was incorporated into the heterokaryon while the cytoplasm was lost by lysis (2, 3). Erythrocytes from 3 to 5-day chick embryos could be fused with other cells with retention of erythrocyte cytoplasm (4); but since the degree of inactivation of embryonic erythrocyte nuclei is uncertain, they seem to be unsuitable for nuclear activation studies. The present communication describes some of the properties of heterokaryons formed by fusing adult chicken and human erythrocytes with other cells, without loss of erythrocyte cytoplasm. These heterokaryons were formed with the aid of Sendai virus in the presence of bivalent metal ions (5–8).

MATERIALS AND METHODS

Materials

Tricine was obtained from Calbiochem, San Diego, Calif. Fetal calf serum was purchased from Microbiological Associates, Inc., Bethesda, Md. Dulbecco's Modified Eagle's medium and F-10 medium were purchased from Microbiological Associates, Jerusalem, Israel.

Salt Solution

The medium (solution K) used for the suspension of the cells and for the fusion process contained 5.35 mM NaCl, 135 mM KCl, 0.8 mM MgSO4, and 20 mM Tricine-NaOH buffer, pH 7.8.

Cells

Chicken embryo erythrocytes were collected under sterile conditions from the chorioallantoic blood vessels of 12–14-day chick embryos. Adult chicken erythrocytes were obtained as described previously (6). Human blood, type O, aged 4–8 wk was used in experiments. The blood cells of each type were washed three times with solution K. The buffy layer containing white cells was discarded and the pellet was suspended in solution K to give a concentration of 5% (vol/vol). Ehrlich ascites tumor cells were obtained as described previously (9) and suspended at a 10% (vol/vol) concentration in solution K.

Cells of a subclone 3460-3B of a Syrian hamster melanoma cell line (10) were the generous gift of Drs. B. Ephrussi and M. C. Weiss (Centre de Génétique Moléculaire, CNRS, 91, Gif-sur-Yvette, France). The cells were grown in glass T flasks containing F-10 med-

FIGURE 1 Phase contrast micrograph of chicken erythrocyte-Ehrlich ascites cell heterokaryons. A sample containing 0.2 ml of Ehrlich ascites cell suspension 10% (vol/vol) plus 0.3 ml of 5% (vol/vol) chicken erythrocyte suspension was preincubated as described in Materials and Methods and the volume made up to 1.0 ml with solution K containing MnCl2 (final concentration 2 mM) and 2400 HAU (hemagglutinating units) of Sendai virus before fusion. A, Ehrlich ascites cell; E, erythrocyte polykaryon; H, heterokaryon. × 560.
FIGURE 2  Electron micrographs of chicken erythrocyte-Ehrlich ascites cell heterokaryons. (a) Heterokaryon (H), unfused Ehrlich ascites cell (A), and unfused erythrocytes (E). Cells were fused with 400 HAU/ml of Sendai virus and 4 mM MnCl₂ as described under Fig. 1. Unfused Ehrlich ascites cells were added after fusion to compare the density of their cytoplasm with that of heterokaryon cytoplasm. Note that both the cytoplasm and the nucleoplasm of the heterokaryon are denser than cytoplasm and nucleoplasm of the Ehrlich ascites cell. × 4,916. (b) Cytoplasmic bridge formation. Cells were fused with 2460 HAU/ml of Sendai virus and 4 mM MnCl₂. The arrows show cytoplasmic bridges. × 9,700.
ium supplemented with 10% fetal calf serum in an atmosphere of 95% air and 5% CO₂. The cells were harvested either by trypsinization with 0.25% trypsin in phosphate-buffered saline or by gentle scraping with a sterile magnet and suspended in fresh growth medium. The cells were washed twice with solution K (180 g for 7 min) and suspended in solution K at a density of about 10⁷ cells/ml.

**Virus**

Sendai virus was isolated and its hemagglutinin titer was determined as described previously (5).

**Cell Fusion**

The cell suspension in solution K (0.5 ml) was incubated in 25-ml scintillation counter vials for 30 min at 37°C in a rotatory shaking bath at 100 rpm. The vials were cooled in ice, and after making up the volume to 1.0 ml with solution K containing virus and other appropriate additions, agglutination was allowed to proceed at 0°C for 10 min. The suspension was then shaken at 37°C for 1 h to facilitate fusion, and finally, cooled in ice. The fusion index (11) and the degree of hemolysis were measured as described previously (6).

**Preparation of Samples for Light and Electron Microscopy**

For light microscopy, samples were fixed in methanol for 10 min, washed in water, and dried. The cover slips were then covered with Giemsa stain, diluted 1:25 for 20 min, washed in water, and dried. Hemoglobin was detected histochemically by a modification of the method of Ralph (12). Samples were prepared for electron microscopy as described previously (9).

**RESULTS**

**Fusion of Chicken Erythrocytes with Ehrlich Ascites Tumor Cells**

Efficient heterokaryon formation with a high fusion index and a low degree of hemolysis (not more than 4%) was obtained with Sendai virus in the presence of suitable concentrations (1–4 mM) of MnCl₂(8). Fig. 1 shows a phase micrograph of heterokaryons of chicken erythrocytes and Ehrlich ascites tumor cells formed under these conditions. The heterokaryons contain nuclei and cytoplasm of both cell types. The presence of hemoglobin in the heterokaryons was confirmed by specific staining with the benzidine stain (12).

Electron micrographs show cytoplasmic bridges which are formed between erythrocytes and Ehrlich ascites tumor cells during the early stages of fusion (Fig. 2). Because of the high ratio of erythrocytes to Ehrlich ascites cells, the latter surround the former. In the heterokaryons, a uniform cytoplasm whose density is close to that of the erythrocyte cytoplasm and markedly denser than that of Ehrlich ascites cells is observed. This is interpreted to mean that the heterokaryons contain erythrocyte cytoplasm and that complete mixing has occurred. It should be noted that the nucleoplasm of Ehrlich ascites cell nuclei in the heterokaryons is more electron dense than that in control cells (Fig. 2 a). This indicates that the erythrocyte cytoplasm penetrates freely into the nuclei of the Ehrlich ascites cells.

**FIGURE 3 Phase contrast micrographs of giant cells formed by fusion of human erythrocytes and Ehrlich ascites cells. The picture was taken 5 min after fusion. A sample containing 0.3 ml of 10% (vol/vol) Ehrlich ascites cell suspension and 0.2 ml of 5% (vol/vol) human erythrocyte suspension was preincubated as described above. The volume was made up to 1.0 ml with solution K containing 1640 HAU of Sendai virus and UO₂(CH₃COO)₂ (final concentration 1.4 mM), and the fusion procedure was continued. G, giant cell; E, unfused erythrocytes; A, unfused Ehrlich ascites cell; P, hemolyzed polyerythrocyte. X 560.**

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FIGURE 4 Electron micrograph of a giant cell resulting from the fusion of human erythrocytes and Ehrlich ascites cells. Fusion conditions: 1.3 mM UO₂(CH₃COO)₂, 1640 HAU/ml Sendai virus. Cells were fixed immediately after fusion. The outline of erythrocyte (E) which was fused with the Ehrlich ascites cell can be clearly seen in the giant cell. X 9,065.

FIGURE 5 Light micrographs of chicken erythrocyte-melanoma heterokaryons after different periods of culture. The fusion system containing 7.5 X 10⁶ melanoma cells and 7 X 10⁷ chicken embryo erythrocytes in 0.35 ml was preincubated as described in Materials and Methods. The volume was made up to 0.5 ml with solution K, containing MnCl₂ (final concentration 4 mM) and 800 HAU of Sendai virus inactivated with UV according to Harris et al. (2). After standing at 0°C for 10 min, the vials were shaken at 37°C at 75 rpm for 45 min. After fusion the cells were sedimented by centrifugation at 180 g for 7 min and suspended in 1 ml of growth medium (F-10 plus 10% fetal calf serum). Portions of the cell suspension were diluted 10-, 10⁻²-, 10⁻⁵-, and 10⁻⁷-fold with growth medium. Samples containing 0.2 ml diluted cell suspension were placed on sterile cover glasses in plastic petri dishes 6 cm in diameter. The petri dishes were incubated overnight at 37°C to permit attachment of cells to the cover glasses. 4 ml of growth medium were then added to each petri dish. Heterokaryons were maintained in culture for 7 days. Every day cover glasses with attached heterokaryons were removed for staining and microscopic examination. (a) Heterokaryon (H) immediately after fusion alongside unfused melanoma cell (M). (b) Heterokaryon containing five melanoma nuclei and nine erythrocyte nuclei, 1 day after fusion. (c) Heterokaryon containing four melanoma nuclei and five erythrocyte nuclei, 5 days after fusion. (d) Heterokaryon containing three melanoma nuclei and five erythrocyte nuclei with well-defined nucleoli, 7 days after fusion. X 1,400.
Fusion of Human Erythrocytes with Ehrlich Ascites Tumor Cells

Efficient fusion of human erythrocytes with Ehrlich ascites cells with little (10-15%) hemolysis was observed with Sendai virus in the presence of about 1.5 mM UO2++ (7, 8). Fig. 3 shows the products of fusion of Ehrlich ascites cells and human erythrocytes which contain Ehrlich ascites cell nuclei and the cytoplasm of both cell types. Since the fusion was performed with an excess of erythrocytes, no homopolykaryons of Ehrlich ascites cells are observed. However, the products of fusion of several erythrocytes ("polyerythrocytes") may be seen (Fig. 3). Fig. 4 shows an electron micrograph of an erythrocyte fused with an Ehrlich ascites cell and fixed 45 min after addition of virus. Although the outline of the erythrocyte is still clearly visible, the cytoplasms of the two cells seem to have mixed. This was deduced from the fact that the density of the cytoplasm of the fused erythrocyte was less than that of unfused erythrocytes but greater than that of Ehrlich ascites cells. Here, as in the case of chicken erythrocyte-Ehrlich cell heterokaryons, the high electron density of the nucleoplasm suggests that the erythrocyte cytoplasm penetrates into the Ehrlich ascites cell nuclei.

Activation of Erythrocyte Nuclei in Melanoma-Chicken Erythrocyte Heterokaryons

Fusion of chicken erythrocytes with melanoma cells was achieved with UV inactivated Sendai virus under conditions similar to those used for fusion with Ehrlich ascites cells. Fig. 5 a shows giant heterokaryons of chicken and melanoma cells in suspension immediately after fusion. Both staining with benzidine (12) and quantitative measurements showed that hemoglobin was present in the cultured heterokaryons for at least 2 days and thereafter decreased in amount. Figs. 5 b, c, and d show heterokaryons cultured for different periods. It is clear from the pictures that the erythrocyte nuclei swell progressively during culture. Nucleoli were discernible in erythrocyte nuclei in some of the cells between the second and third day of culture and clearly visible in most erythrocyte nuclei after 7 days in culture.

After 7 days' incubation, the diameter of the erythrocyte nuclei in the heterokaryons increased 3-4-fold (Fig. 6). Already 1 day after fusion there was a significant increase in the diameter of the erythrocyte nuclei. The degree of swelling of the erythrocyte nuclei was markedly affected by the total number of erythrocyte nuclei present in the heterokaryon. When only a single erythrocyte nucleus was present in the heterokaryon, both the initial rate of activation and the final diameter of the nucleus was significantly higher than when two or more nuclei were incorporated (Fig. 6).

Increasing the number of erythrocyte nuclei per heterokaryon, without changing the ratio of erythrocyte to melanoma nuclei, resulted in slower nuclear swelling (Table I). It was not clear, however, from the results in Table I whether or not the rate of swelling of erythrocyte nuclei decreased with increasing ratio of erythrocyte to melanoma nuclei.

DISCUSSION

The above data show the results of fusing adult chicken and human erythrocytes with other types...
The diameter of erythrocyte nuclei was measured after 5 days in culture in heterokaryons having different total numbers of erythrocyte nuclei but similar ratios of erythrocyte to melanoma nuclei. The method of measuring nuclear diameter and the arbitrary units used were the same as in Fig. 6. The mean diameters of erythrocyte nuclei within each cell were first calculated, and then the statistical analysis was performed on these mean values as shown in Table I. In heterokaryons with a nuclear ratio of approximately 1, there was a significant difference in nuclear diameter (F test) between groups A and (B + C) (P < 0.01), between groups (B + C) and D (P = 0.001), and between Groups A and D (P < 0.001). There was no significant difference between groups B and C. In heterokaryons with a nuclear ratio (E/M) ~ 2-3, there was a significant difference between groups B and C (P < 0.01) and between groups B and D (P < 0.001). There was no significant difference between groups C and D. In group B and in group D there was no significant difference between nuclear ratios 1 and 2-3. In group C the diameter of nuclei in cells with nuclear ratio 1 was significantly greater than that in heterokaryons with nuclear ratio 2-3 (P < 0.001).

* E/M = Erythrocyte nuclei/melanoma nuclei.

Table I

| Group | Number of erythrocyte nuclei | Ratio E/M | Number of cells | Mean diameter ± SD | Ratio E/M | Number of cells | Mean diameter ± SD |
|-------|-----------------------------|-----------|----------------|-------------------|-----------|----------------|-------------------|
| A     | 1:1                         | 10        | 15.6 ± 3.6     | 2:1               | 4         | 12.5 ± 0.9     |
| B     | 2:2                         | 5         | 12.1 ± 1.9     | 4:2               | 4         | 9.1 ± 1.4      |
| C     | 3:3                         | 1         | 12.2 ± 1.4     | 5:3               | 5         | 9.4 ± 1.0      |
| D     | 6-15                        |           |                | 7:6               | 1         | 11.7 ± 1.1     |
|       |                             |           |                | 7:5               | 2         | 10.8 ± 1.1     |
|       |                             |           |                | 8:7               | 2         | 10.3 ± 1.1     |
|       |                             |           |                | 8:6               | 10        | 9.9 ± 1.1      |
|       |                             |           |                | 9:7               | 1         | 8.5 ± 1.3      |
|       |                             |           |                | 10:8              | 2         | 9.4 ± 1.1      |
|       |                             |           |                | 14:11             | 1         | 9.3 ± 1.1      |

The experiments with melanoma-chicken erythrocyte heterokaryons show that the inclusion of erythrocyte cytoplasm in the heterokaryon does not interfere with the reactivation of the erythrocyte nucleus as indicated by nuclear swelling and nucleolus formation (1, 15). These results are consistent with the observations of Harris and his colleagues who found that in macrophage-HeLa and lymphocyte-HeLa heterokaryons, in which the cytoplasm of both cell types are mixed, DNA synthesis in the lymphocyte and macrophage nuclei was reactivated (2). The rate and extent of nuclear swelling decreased markedly with the increasing number of erythrocyte nuclei in the heterokaryons (Fig. 6). Previous investigations (16, 17) have shown that the rate of reactivation of chicken erythrocyte nuclei in heterokaryons decreased with the increasing ratio of chicken erythrocyte to host cell
nuclei. Carlsson, Moore, and Ringertz (17) suggest that “erythrocyte nuclei compete with each other for molecules which are of specific importance for the reactivation process.” It should be noted that in both previous studies, fusion was carried out under conditions which result in the loss of erythrocyte cytoplasm. The present study supports the assumption that there are factors activating erythrocyte nuclei in host cell cytoplasm, and further shows that the effect of these factors is not neutralized by the inclusion of erythrocyte cytoplasm. An observation which remains paradoxical is that the rate of nuclear swelling in our studies decreased with the absolute number of erythrocyte nuclei per heterokaryon and apparently not according to their ratio to host cell nuclei (Table I). This observation seems to be incompatible with a simple competition by erythrocyte nuclei for activation factors and requires further clarification.

SUMMARY

In the presence of Sendai virus and bivalent cations, chicken and human erythrocytes fused with Ehrlich ascites tumor cells with almost complete retention of erythrocyte cytoplasm. Electron micrographs indicate that mixing of erythrocyte and Ehrlich ascites cell cytoplasm is rapid after fusion and that erythrocyte cytoplasm penetrates freely into the Ehrlich ascites cell nuclei. During culture of melanoma-chicken erythrocyte heterokaryons containing the cytoplasm of both cell types, the erythrocyte nuclei increased in size and nucleoli were formed.

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