GIANT CULTURE CELLS BY ELECTRIC FIELD-INDUCED FUSION

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1. Introduction

Because of the many potential applications in cell membrane research, genetic mapping and somatic hybridization (e.g., production of hybridoma cells), the development of techniques for in vitro cell fusion is receiving ever increasing attention [1–5]. However, cell fusion induced by the usual means (e.g., glycerol-monooleate, polyethylene glycol, Sendai virus), suffers from several disadvantages, i.e., unphysiological conditions, lack of control of the fusion process under the microscope, large variability in the number of cells subjected to fusion, low yield of fused cells, loss of intracellular components and limited viability. A new method for cell-to-cell fusion based on electrical breakdown in the membrane contact zones of two cells attached to each other has been introduced [6–14]. This method eliminates most of the disadvantages of the chemical- and virus-induced fusion techniques. Electric field-induced cell-to-cell fusion is performed in two steps.

(1) Tight membrane contact between cells is achieved by dielectrophoresis [15,16], i.e., by movement of the cells under the influence of a non-uniform, alternating electric field of low intensity. This results in the formation of so-called pearl chains, the length of which depends on the cell suspension density and the inhomogeneity of the field.

(2) Fusion between cells in a pearl chain is induced by an additional external field pulse of short duration and high intensity which results in the reversible electrical breakdown of the cell membrane [10,17,18]. Since the dielectrophoretically collected cells are aligned parallel to the field, electrical breakdown predominantly occurs in the contact zone between any two cells. After breakdown, the fusion process takes place within seconds to several minutes, depending on the species.

This technique has been successfully applied in the fusion of plant protoplasts of different species, sea urchin eggs and human erythrocytes [6–14]. In the latter case giant fused cells of up to 1 mm diam. were obtained.

Here, we report on electric field-induced fusion of a permanent mammalian cell line in order to test the potential of this method for somatic hybridization and for the production of giant cells from mammalian cells which could then be impaled with microelectrodes. Friend cells (erythroblasts obtained by transformation with Friend virus) were used because the biochemical activity and, in turn, viability of the fused cells could easily be determined by means of the dimethylsulfoxide (DMSO)-induced haemoglobin synthesis.

2. Materials and methods

Murine virus-induced leukemic cells (Friend cells) [19] were obtained by courtesy of Professor Goebel, Würzburg and cultivated in a modified basal Eagle's medium. For the fusion experiments cells were collected from suspensions of $\sim 10^5$ cells/ml. All steps were carried out under sterile conditions. The cells were centrifuged and the pellet resuspended in 0.3 M mannitol solution. In general, the cells were washed twice with mannitol solution before subjecting to dielectrophoresis.

The experimental set-up for electric field-induced cell fusion is detailed in [8]. The fusion chamber consisted of a slide with two parallel platinum wires glued to it at a minimum distance apart of 200 μm. The wires, which served as electrodes, had a diameter of

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200 μm. Experiments were carried out under a microscope with a long-working-distance objective, while the fusion chamber was maintained at 20°C. The alternating field for dielectrophoresis of the cells was generated by a function generator (Toellner type TE 7704, M. Werner, Frankfurt).

Breakdown in the membranes of the aligned cells was induced using a pulse generator (Hewlett Packard type 214 B). Both devices were connected in parallel to the electrodes. A switch disconnected the function generator from the electrodes for 1 ms when the breakdown pulse was applied. For a fusion experiment, a 100 μl droplet of 0.3 M mannitol solution was pipetted between the electrodes of the fusion chamber and a few μl of cell suspension were additionally injected into the gap between the electrodes.

The DMSO-induced haemoglobin stimulation was performed according to [19]. The induction of heamoglobin synthesis was demonstrated by the benzidine reaction [20].

3. Results and discussion

Friend cells in logarithmic growth phase were exposed to the alternating, slightly non-uniform field. The intensity of the field was ~0.1 kV/cm and the frequency chosen between 100 kHz and 2 MHz. Lower frequencies were not used because of the rotation phenomenon observed in Friend cells at a frequency of ~40 kHz [21], which disturbs the alignment of the cells along the field lines and prevents close membrane contact. Field strengths exceeding the breakdown voltage must be avoided during dielectrophoretic collection of cells because electrical breakdown of the cell membranes immediately results in the mechanical destruction of the cells under these conditions [6,22].

The critical field strength required for reversible electrical breakdown can be calculated on the basis of the integrated Laplace equation derived for spherical cells [23]:

$$V_c = 1.5 \cdot r \cdot E_c \cdot \cos\theta$$

E_c is the critical field strength and θ is the angle between a given membrane site and the field direction. V_c = 0.85 V is the breakdown voltage [24] and r = 8 μm is the radius of the cells [24]. Using these values the critical field strength for the two membrane sites in the field direction, i.e., in the membrane contact zone ($\cos\theta = 1$), is calculated to be ~0.7 kV/cm. In the derivation of the integrated Laplace equation it is assumed that the field is uniform and that the stationary membrane potential corresponding to a given external field strength is established. As pointed out above, the field is slightly non-uniform and the relaxation time of the charging process of the membrane can be of several μs in the presence of a non-electrolyte solution. Thus, the value of 0.7 kV/cm for the field strength is only a rough estimate. In any case, the presence of the alternating field causes many breakdown events to occur within the membranes, if the critical field strength is exceeded and if there is insufficient resealing time between two breakdown events; i.e., if the frequency is too high, the resealing time of cell membranes is in general of the order of several minutes [6,10]. The reversible electrical breakdown event will thus go over into the mechanical destruction of the cell, as observed in suspended cells and artificial planar lipid bilayer membranes in response to a single field pulse of very high intensity and long duration (so-called supercritical field pulse; for definition see [6]).

Fig.1 shows 'pearl chains' of Friend cells obtained by dielectrophoresis in an alternating field of low intensity. Fusion was initiated by the application of a square field pulse of 40 V amplitude and 20 μs duration. In general, 2–3 cells fused together within 3 min, although a number of cells deteriorated. The alternating voltage was reduced to almost zero, once the process of intermingling of the 2 membranes became visible under the microscope. Transfer of the fused cells into isotonic electrolyte solution enhanced

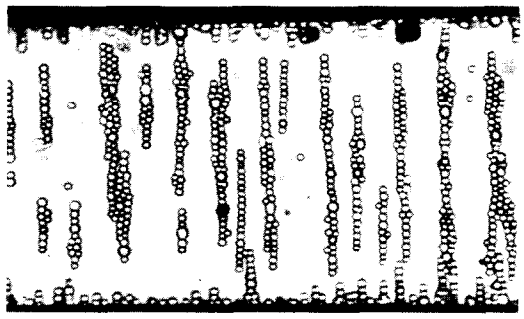

Fig.1. Dielectrophoretic collection of Friend cells. The cells were suspended in 0.3 M mannitol solution and placed between the electrodes. After application of an inhomogeneous alternating field (100 V/cm, 2 MHz) the cells arrange like pearl chains.

the formation of spherical cells. The yield was ~20–30%, provided the cells were taken from the logarithmic phase and incubated for only a very short time in the non-electrolyte solution.

The yield of fused cells could be considerably increased if pronase or dispase were added to the cells just before dielectrophoresis. In the presence of these enzymes cells could also be subjected to fusion after a longer incubation time (up to 4 h) in the non-electrolyte solution. In the presence of 1 mg pronase/ml or 10 μg dispase/ml, up to 100% of the cells fused within 2–3 min. The size of the fused cells could be preselected by using different cell suspension densities. At low suspension densities pearl chains of 2–3 cells predominate; thus the probability of obtaining hybrids is high. On the other hand, a high yield of giant cells (up to 200 μm diam.) was obtained, if high suspension densities were used. Under these conditions many pearl chains are arranged in parallel to each other, and close membrane contact is established between cells of parallel chains [11]. The applied field strength of 4 kV/cm is sufficiently high to induce breakdown in those membrane areas orientated at an angle of ~80° with respect to the field direction (see the Laplace equation). Thus fusion can take place not only between cells within a given pearl chain (i.e., in the field direction), but also between cells of adjacent pearl chains (almost parallel to the electrode). Obviously, this procedure leads to the formation of giant cells (see fig.2). The giant cells were mechanically stable and could easily be transferred to other solutions. Dispase has the considerable advantages over pronase that smaller concentrations are needed and it is milder in its reaction. It is well known that permanent cell lines can be grown in the presence of small amounts of dispase [25,26].

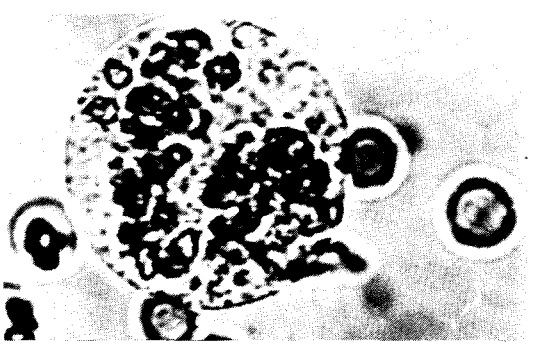

Fig.2. Giant cell obtained by fusion of Friend cells. For fusion the cells were suspended in 0.3 M mannitrol solution containing 1 mg pronase/ml and collected by dielectrophoresis (100 V/cm, 2 MHz). After 1 min a breakdown pulse with a field strength of 2 kV/cm and a duration of 20 μs was applied. The photograph was taken 5 min after the pulse.

The presence of the enzymes apparently stabilises the cells against the field strength [27]. The field strength of the alternating field and of the field pulse, respectively, which could be applied to the cells without deterioration, increased with increasing enzyme concentration and with increasing exposure time to the enzymes. At 1 mg pronase/ml or at 10 μg dispase/ml (exposure for 10 min) the cells could be subjected to a field pulse of 5 kV/cm and 100 μs duration without deterioration. These field conditions resulted in a very rapid fusion of the cells in the pearl chains. The fusion process was almost complete within a couple of seconds. Removal of the enzymes by washing the treated cells several times with enzyme-free non-electrolyte solution resulted in the well-known field-sensitivity of the cells. Thus, we can conclude that the enzyme effect on the membrane is completely reversible, provided that the exposure time of the cells to the enzyme does not exceed 30 min.

The viability of the fused cells apparently depended on the speed of the fusion process and thus on the field strength and the duration of the field pulse. It seems that fusion must proceed for 2–3 min after the breakdown pulse has been applied, to obtain viable cells. The optimum conditions for enzyme treatment and field application are given in fig.2. A high yield of fused Friend cells in the absence of pronase can also be achieved by subjecting the cells to a train of field pulses of increasing field strength and duration, beginning with a field strength of 2 kV/cm and a duration of 5 μs and ending up with a field strength of 5 kV/cm and a duration of 50 μs. There is experimental evidence that Friend cells can also be subjected to pulses of higher field strength and longer duration under these conditions without the deterioration that would normally be the case. Apparently, this treatment also stabilises the cells against high field strengths and long pulse lengths. The viability of the fused cells was tested by DMSO-induced haemoglobin synthesis. The stimulation of haemoglobin synthesis in fused cells required in average 2 days, as with cells not subjected to fusion. The benzidine reaction revealed that almost 100% of the fused cells could synthesise heamoglobin [19] (fig.3).

Measurements of the electrical breakdown voltage

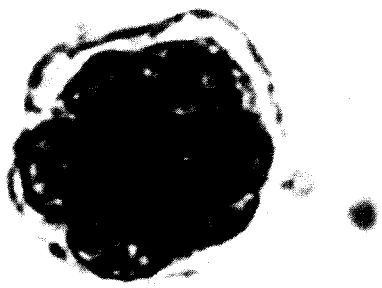

Fig.3. Haemoglobin stimulation in a giant cell obtained by fusion of Friend cells. The fusion was performed under the same conditions as in fig.2. For stimulation of the haemoglobin synthesis the giant cells were washed 3 times with nutrient medium to remove the pronase and transferred into basal Eagle's medium containing 2% DMSO. After 3 days haemoglobin synthesis was demonstrated by the benzidine reaction.

of Friend cells, using a single particle analyser [24], revealed no significant differences in the breakdown voltages of Friend cells in the absence or presence of the enzymes. In all cases the breakdown voltage was ~0.85 V. The apparent discrepancy between the unchanged breakdown voltage of the cell membranes in the presence of the enzymes, on the one hand, and the higher field stability, on the other hand, can be explained in terms of the emergence of protein-free lipid domains in the membrane contact zone. In [28] the redistribution and aggregation of intramembranous particles (which are probably of proteins) in virus-induced and other types of membrane fusion was proposed to serve as a mechanism of depleting areas of the membrane of integral proteins. This idea is consistent with experimental data (see, e.g. [1]). In addition to proposing that membrane proteins aggregate during membrane fusion, it was also suggested that fusion might proceed by the interdigitation of the aggregated particles [2,28]. However, in [1,29] it was postulated that fusion proceeds by the intermingling of membrane lipids after protein-free areas of lipid bilayer have emerged as a result of protein aggregation.

Considering the above results we suggest that according to [1,29] particle-free lipid domains are a prerequisite for the intermingling of two membranes (fig.4). We postulate that the enzymes we used lead to aggregation of particles and, in turn, to the emergence of lipid domains whereby the alternating electric field seems to enhance aggregation. Aggregation of particles in the presence of proteolytic enzymes has been observed before but the only recorded experiments were performed at higher temperatures (>40°C) with long exposure times to the enzyme (60 min) (see [29]). In contrast, the field stabilisation effects of the enzymes on the membranes of Friend cells reported here were recorded at room temperature

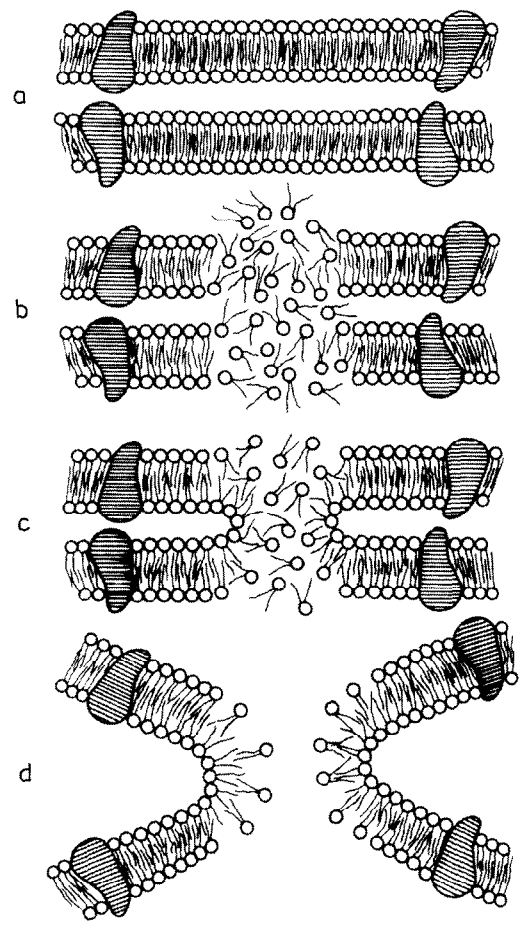

Fig.4. The lipid bilayers between the proteins of the two membranes are in close contact vertical to the field lines (a), whereby it is assumed that protein-free areas are formed by lateral diffusion of proteins. Electrical breakdown leads to a disruption of the membrane structure (b), so that bridges may be formed between the lipid bilayers of the two membranes during the reorganisation of the lipid molecules (c). The radius of curvature of the pores formed in this way is very small, so that formation of spherical 'two-celled aggregates' is energetically favored (d).

within 30 s of adding the enzymes and were completely reversible.

Either pronase or dispase may enhance lateral movement of the intramembranous particles in response to the field by degradation of a few integral proteins in the membrane contact zone [29,30,31], or the activity of these enzymes may be changed in the presence of the electric field and the non-electrolyte environment [27]. In any case, the only straightforward explanation for the 'field insensitivity' of the cells in the presence of these enzymes is the formation of lipid domains in the membrane contact zones. From electrical breakdown studies on artificial planar lipid bilayer membranes we know that breakdown occurs in <10 ns and that the membrane voltage decays to very low levels within hundreds of ns [32]. The resealing time of the membrane which has been subjected to electrical breakdown is <2 μs [33]. However, in cell membranes electrical breakdown most probably occurs at the lipid–protein junction or within the proteins [34]. Therefore field-induced effects in proteins require a much longer resealing time (up to 30 min, depending on the temperature [6,17]). Due to the high conductance and permeability state of the membranes for a long period, the field lines will partly pass through the cell interior and cause adverse side effects if the field strength exceeds a certain level or if the field pulse is longer than say 2–5 μs [6,10,17]. In addition to the field effects on the cells, secondary processes (e.g., osmotic) can lead to irreversible destruction of the cells, if breakdown has not occurred in the lipid domains, as in the case of cells exposed to the field in the presence of enzymes [6,10,17].

The proposed mechanism for electric field-induced fusion is shown in fig.4. Breakdown in the lipid domains leads to the formation of pores as a result of local electro-mechanical compression of the membrane [19]. The randomly orientated lipid molecules within the pore bridge the two apposed bilayer under the formation of a channel as indicated (fig.4). We have to assume that this process has a much faster time constant than that of the resealing process of the lipid bilayer itself. However, if the time constant of the bridging process is slower (e.g., because of the presence of some integral proteins at the site of breakdown) the resealing process of the bilayer membrane becomes the dominant one and fusion will not occur. When the channel is formed, high curvature and, in turn, the high tension in the two bridged bilayers bring about the formation of a sphere which is the only thermodynamically stable configuration. Osmotic processes (as postulated [35]) for chemical- and virus-induced fusion do not seem to be involved. The stabilisation of cells against field pulses of high field strength and long duration by prior injection of a train of field pulses of lower intensity and shorter duration can also be interpreted in terms of the proposed mechanism. The generation of pores will cause the membrane to reseal. There is a very high probability that the proteins and lipids become separated since the diffusion coefficient of the lipids is higher by 2 orders of magnitude than that of the proteins [36]. Thus, it is plausible to assume that the pores created by breakdown are closed by lipids rather than by proteins. The hypothesis that fusion is only initiated when breakdown occurs in lipid domains is further supported by the finding that fusion between ATP-depleted human erythrocytes is observed both in the absence of pronase or neuraminidase during field application and without enzymatic pretreatment of the cells before field application [37]. With ATP-depleted cells, giant cells of 100 μm diam. can be obtained provided that EDTA prevents uptake of Ca^{2+} during the fusion process. Electronmicrographs showed that ATP-depletion leads to the emergence of lipid domains [37].

The above procedure yields thousands of fused cells, and considerably more if it is repeated several times or if a flow chamber is used [8]. The number of fused cells obtained by the present set-up should be sufficient for applications in medicine and membrane research. Since the fusion process can be followed under the microscope, the fused cells can easily be identified, particularly if cells of different species are subjected to electric field-induced fusion.

Preliminary experiments with B-lymphocytes from mouse spleen and myeloma cells have shown that the fusion conditions reported here can be applied to these cells without modification. Thus it should be possible, in the very near future, to obtain well-defined hybridoma cells which have great potential in clinical diagnosis and therapy as well as in the purification and enrichment of compounds like interferon (e.g., [4,5,38,39]).

The formation of giant cells in high yield will permit direct measurements of the electrical membrane properties by insertion of microelectrodes into these cells. This procedure may open the possibility of comparing the membrane properties of normal and malignant cells.

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References

[1] Lucy, J. A. (1978) in: Cell Surface Reviews (Poste, G. and Nicolson, G. L. eds) vol. 5, pp. 267–304, Elsevier/North-Holland, Amsterdam, New York.
[2] Poste, G. and Pasternak, C. A. (1978) in: Cell Surface Reviews (Poste, G. and Nicolson, G. L. eds) vol. 5, pp. 305–367, Elsevier/North-Holland, Amsterdam, New York.
[3] Power, J. B., Evans, P. K. and Cocking, E. C. (1978) in: Cell Surface Reviews (Poste, G. and Nicolson, G. L. eds) vol. 5, pp. 369–385, Elsevier/North-Holland, Amsterdam, New York.
[4] Köhler, G. and Milstein, C. (1976) Eur. J. Immunol. 6, 511–519.
[5] Olsson, L. and Kaplan, H. S. (1980) Proc. Natl. Acad. Sci. USA 77, 5429–5431.
[6] Zimmermann, U., Vienken, J. and Pilwat, G. (1980) Bioelectrochem. Bioenerg. 7, 553–574.
[7] Zimmermann, U., Vienken, J. and Scheurich, P. (1980) Biophys. Struct. Mech., vol. 6, suppl., 86.
[8] Zimmermann, U. and Scheurich, P. (1980) Planta 151, 26–32.
[9] Zimmermann, U. and Scheurich, P. (1981) Biochim. Biophys. Acta 641, 160–165.
[10] Zimmermann, U., Scheurich, P., Pilwat, G. and Benz, R. (1981) Angew. Chem. 93, 332–351; Angew. Chem. Int. Ed. Engl. 20, 325–344.
[11] Scheurich, P. and Zimmermann, U. (1981) Naturwissenschaften 68, 45–46.
[12] Scheurich, P., Zimmermann, U. and Schnabl, H. (1981) Plant Physiol. 67, 849–853.
[13] Vienken, J., Ganser, R., Hampp, R. and Zimmerman, U. (1981) Plant Physiol., in press.
[14] Richter, H.-P., Scheurich, P. and Zimmermann, U. (1981) Devel. Growth Diff. 23, in press.
[15] Pohl, H. A. (1978) Dielectrophoresis, Cambridge University Press, Cambridge.
[16] Schwan, H. P. and Sher, L. D. (1969) J. Electrochem. Soc. 116, 22–26.
[17] Zimmermann, U., Pilwat, G., Beckers, F. and Riemann, F. (1976) Bioelectrochem. Bioenerg. 3, 58–83.
[18] Zimmermann, U., Pilwat, G. and Vienken, J. (1980) Rec. Res. Cancer Res. 75, 252–259.
[19] Friend, C., Scher, W., Holland, J. G. and Sato, T. (1971) Proc. Natl. Acad. Sci. USA 68, 378–382.
[20] Rick, W. (1972) in: Klinische Chemie und Mikroskopie, pp. 280–281, Springer-Verlag, Berlin, New York.
[21] Zimmermann, U., Vienken, J. and Pilwat, G. (1980) Z. Naturforsch. 36c, 173–177.
[22] Benz, R., Beckers, F. and Zimmermann, U. (1979) J. Membr. Biol. 48, 181–204.
[23] Jeltsch, E. and Zimmermann, U. (1979) Bioelectrochem. Bioenerg. 6, 349–384.
[24] Zimmermann, U., Groves, M., Schnabl, H. and Pilwat, G. (1980) J. Membr. Biol. 52, 37–50.
[25] Freimueller, B., Mueller, H. W. and Lamprecht, W. (1977) 11th FEBS Meeting Copenhagen, August 1977.
[26] Ono, J., Takaki, R. and Fukuma, M. (1977) Endocrinol. Japan 24, 365–370.
[27] Zimmermann, U., Richter, H.-P. and Pilwat, G. (1981) Naturwissenschaften, in press.
[28] Poste, G. (1974) Int. Rev. Cytol. 33, 157–252.
[29] Ahkong, Q. F., Blow, A. M. J., Bothman, G. M., Launder, J. M., Quirk, S. J. and Lucy, J. A. (1978) FEBS Lett. 95, 147–152.
[30] Passow, H. (1971) J. Membr. Biol. 6, 233–258.
[31] Passow, H., Fasold, H., Lepke, S., Pring, M. and Schuhmann, B. (1977) in: Membrane Toxicity (Miller, M. W. and Shamoo, A. E. eds) pp. 353–379, Plenum Press, London, New York.
[32] Benz, R. and Zimmermann, U. (1980) Biochim. Biophys. Acta 597, 637–642.
[33] Benz, R. and Zimmermann, U. (1981) Biochim. Biophys. Acta 640, 169–178.
[34] Zimmermann, U., Beckers, F. and Coster, H. G. L. (1977) Biochim Biophys. Acta 464, 399–416.
[35] Zimmerberg, J., Cohen, F. S. and Finkelstein, A. (1980) Science 210, 906–908.
[36] Webb, D. (1978) in: Electrical Phenomena at the Biological Membrane Level (Roux, E. ed) pp. 119–153, Elsevier/North-Holland, Amsterdam, New York.
[37] Saleemuddin, M., Vienken, J. and Zimmermann, U. (1981) Biochim. Biophys. Acta, in press.
[38] Gupta, S. K. and Talwar, G. P. (1980) Ind. J. Exp. Biol. 18, 1361–1365.
[39] Staehlin, T., Durrer, B., Schmidt, J., Takacs, B., Stocker, J., Miggiano, V., Stähli, C., Rubinstein, M., Levy, W. P., Hershberg, R. and Pestka, S. (1981) Proc. Natl. Acad. Sci. USA 78, 1848–1852.