

Cell Adhesion Molecules: Detection with Univalent Second Antibody

WAYNE R. SPRINGER and SAMUEL H. BARONDES

*Department of Psychiatry, Veterans Administration Medical Center, San Diego, California 92161; and
School of Medicine, University of California, San Diego, La Jolla, California 92093*

ABSTRACT Identification of cell surface molecules that play a role in cell-cell adhesion (here called cell adhesion molecules) has been achieved by demonstrating the inhibitory effect of univalent antibodies that bind these molecules in an in vitro assay of cell-cell adhesion. A more convenient reagent, intact (divalent) antibody, has been avoided because it might agglutinate the cells rather than blocking cell-cell adhesion. In this report, we show that intact rabbit immunoglobulin directed against certain cell surface molecules of *Dictyostelium discoideum* blocks cell-cell adhesion when the in vitro assay is performed in the presence of univalent goat anti-rabbit antibody. Under appropriate experimental conditions, the univalent second antibody blocks agglutination induced by the rabbit antibody without significantly interfering with its effect on cell-cell adhesion. This method promises to be useful for screening monoclonal antibodies raised against potential cell adhesion molecules because: (a) it allows for the screening of large numbers of antibody samples without preparation of univalent fragments; and (b) it requires much less antibody because of the greater affinity of divalent antibodies for antigens.

Molecules that bind cells to each other are often referred to as cell adhesion molecules. A number of experimental approaches have been used in an attempt to identify and isolate such molecules (1). An immunological approach has been used with considerable success in studies of cell-cell adhesion in a number of systems, especially cellular slime molds (2-4). Beug et al. (2) and Müller and Gerisch (3) raised antibodies to whole cellular slime molds that had differentiated to the stage where they showed prominent cell-cell adhesion; made univalent antibody fragments (Fab fragments) to eliminate the agglutinating activity of the antibodies that is dependent on divalency; and found that these Fab fragments blocked cell-cell adhesion in an in vitro assay. With this adhesion-inhibition assay, they purified the cell surface molecule to which the active Fab is directed and called it "contact sites A." More recently, a glycoprotein from cellular slime molds, called GP150, has also been identified as a possible cell adhesion molecule by this approach (4). A similar approach has been utilized to identify a glycoprotein from embryonic chick retina which may also play a role in cell-cell adhesion (5).

Recently, techniques for preparing monoclonal antibodies have been successfully developed (6). With these techniques, it is possible to immunize an animal with a crude mixture of antigens, such as a whole cell, and raise discrete antibody

populations that could be specific for a given molecule. In principle, this technique could provide a battery of reagents directed against a variety of cell adhesion molecules. However, screening a large number of such molecules for inhibitory activity in a cell-cell adhesion assay would be very cumbersome if it were necessary to generate univalent antibody fragments from each antibody preparation. Furthermore, the fact that univalent antibody fragments have much lower affinities for antigens than the complete immunoglobulins might mean that fairly large amounts of the monoclonal antibodies would be needed even for initial screening purposes. The relatively small amounts of antibody produced by initial isolates could pose a serious limitation to such an assay. To obviate these potential difficulties, it would be desirable to be able to screen for small amounts of antibodies against cell adhesion molecules using intact immunoglobulins directly prepared by the monoclonal technique. Unfortunately, the effect of divalent antibodies in a cell adhesion assay would be difficult to determine because the divalent antibodies might agglutinate cells bearing appropriate cell surface antigens and actually augment the amount of cell-cell association that was observed. In the present report, we show that when rabbit antibodies raised against putative cell adhesion molecules in cellular slime molds are bound to the surface of these cells, their role in inhibiting cell-cell

adhesion can be evaluated with the use of a second univalent antibody, goat anti-rabbit Fab. With this procedure, the agglutinating effect of the primary antibodies may be overcome and the inhibition of cell-cell adhesion by antibodies directed against putative cell adhesion molecules is clearly demonstrated. In addition, as expected, relatively small amounts of the primary antibody are required. This approach should be useful in screening for putative cell adhesion molecules in cellular slime molds. It also merits consideration in studies with other cellular systems.

MATERIALS AND METHODS

Cells and Culture Conditions

DICTYOSTELIUM DISCOIDEUM: Strain NC-4 was grown in association with *Klebsiella aerogenes*, washed free of bacteria, partially differentiated in suspension for 16 h, and finally developed to tight aggregates on filter-pads, all as described in detail previously (7). These cells are designated "pad developed" and used in the cell-cell adhesion assays described below.

ANTIBODIES: Rabbits were immunized with either whole differentiated (pad developed) *D. discoideum* cells or the carbohydrate binding protein, discoidin, purified from *D. discoideum* as published (8), by an initial intradermal injection in complete Freund's adjuvant followed after a month by five biweekly injections of antigen in incomplete Freund's adjuvant. IgG was prepared from antisera by chromatography on DEAE Affigel Blue (Bio Rad Laboratories, Richmond, Calif.). The IgG raised against differentiated cells is called anti-*D. discoideum* (anti-*Dd*) IgG and the IgG raised against discoidin is called antidisoidin IgG.

IgG prepared against the glycoprotein GPI50 (4) was the kind gift of Dr. John Geltosky of the Scripps Clinic and Research Foundation, La Jolla, Calif., and is referred to as anti-GPI50 IgG.

Fab fragments of goat anti-rabbit IgG, here designated GARFab, were obtained from Cappel Laboratories, Inc., Cochranville, Pa.

CELL-CELL ADHESION ASSAYS: Assays of cell-cell adhesion in the presence or absence of EDTA were described and rationalized previously (9). For the present studies, cells were removed from the filter-pads, washed, and resuspended by vortexing to a concentration of 2×10^7 /ml in cold 16.7 mM $\text{Na}_2\text{PO}_4\text{-KH}_2\text{PO}_4$, pH 6.2 (SPS). 50 μl of this suspension was diluted into 0.5 ml of cold SPS containing rabbit IgG at various concentrations, and the mixture incubated on ice for 30 min. After vigorous vortexing for 15 s to dissociate agglutinated cells, a 50- μl aliquot was removed from the mixture and added to 550 μl of SPS containing 2 mg/ml bovine serum albumin (BSA, RIA grade; Sigma Chemical Co., St. Louis, Mo.) and 0.5 mg/ml GARFab. Depending upon the experiment, the medium also included 10 mM EDTA (Sigma Chemical Co.). At the time of addition of the cells, the medium was in the process of being gyrated in a glass scintillation vial at 200 rpm on a New Brunswick G24 gyratory shaker (19-mm orbit; New Brunswick Scientific Co., Inc., Edison, N. J.). Gyration was continued for 20 min at which time 10 ml of ice-cold 0.15 M NaCl was added and the diluted cell suspension was analyzed in a Coulter electronic particle counter (Coulter Electronics, Hialeah, Fla.) adjusted to count 95% of the single cells. Cell-cell adhesion was measured as the loss of single cells during gyration. The percent cell-cell adhesion was calculated as $1 - (S_t/S_0) \times 100$ where S_t is the number of single cells after gyration and S_0 is the number of single cells added to the vial determined as the number of cells at 0 min where no IgG was added.

It should be emphasized that what we call cell-cell adhesion in this paper is operationally indistinguishable from cell agglutination. By cell-cell adhesion, we mean the binding of cells to each other by endogenous factors. In contrast, by cell agglutination we mean the binding of cells to each other by an exogenous reagent, such as IgG.

¹²⁵I-GARFAB BINDING: GARFab iodinated by the procedure of McConahey and Dixon (10) and separated from unreacted iodine on a Sephadex G-25 column was used to measure the binding of rabbit IgG to slime mold cells. A 200- μl aliquot of cells that had been incubated with rabbit IgG, as in the cell-cell adhesion assay described above, was centrifuged through 1 ml of 10% Ficoll (Sigma Chemical Co.) to separate it from unbound IgG. The cells were then resuspended to the same volume in cold SPS, and 50- μl samples were added to 200 μl of 1.0 mg/ml ¹²⁵I-GARFab (containing 5×10^3 cpm) in SPS with 2 mg/ml BSA. The suspension was shaken and incubated on ice for 1 h. Duplicate 80- μl aliquots were removed from the vortexed suspension, spun through 0.5 ml 10% Ficoll, and the pellet was counted in a gamma scintillation counter. Control cells that were not reacted with rabbit IgG but carried through the remainder of the procedure were also studied and the small number of counts associated with them was subtracted from the counts found associated with the IgG-treated cells.

RESULTS

The IgGs from antibody preparations raised against whole differentiated *D. discoideum* (anti-*Dd* IgG), a purified glycoprotein believed to play a role in cell-cell adhesion (anti-GPI50), and a purified slime mold lectin also implicated in cell-cell adhesion (antidisoidin) all bound extensively to the surface of partially differentiated *D. discoideum* cells (Fig. 1). As would be expected, the greatest amount of IgG binding was observed with the heterospecific antibody (anti-*Dd*). With anti-GPI50, binding was saturable at $\sim 1.2 \times 10^7$ molecules of ¹²⁵I-GARFab/cell, which agrees well with the estimate of cell surface GPI50 of 5.5×10^6 molecules/cell (4). Antidisoidin IgG bound at about the same level as anti-GPI50 IgG and was also saturable. The implications of the finding of such large numbers of endogenous cell surface lectin molecules, when measured by this technique, will be considered in detail elsewhere.¹

The binding studies in Fig. 1 were all performed by binding the IgG to the cells for 30 min at 4°C followed by washing and incubation with ¹²⁵I-GARFab for 1 h. In studies with anti-*Dd* IgG (0.25 mg/ml), we found that binding had reached equilibrium within 15 min and that the amount bound was identical with that bound with 1-h incubations. We also compared ¹²⁵I-GARFab binding at 4° and 24°C, the latter being the temperature for the cell-cell adhesion assay. We found that ¹²⁵I-GARFab binding at 24°C was $\sim 20\%$ higher than at 4°C with both 30- and 60-min incubations.

To evaluate the effects of primary antibodies with or without GARFab on measures of cell-cell adhesion and to establish assay conditions, we performed preliminary studies with anti-*Dd* IgG. We found that when large amounts of anti-*Dd* IgG were bound to differentiated *D. discoideum* cells, there was extensive cell agglutination. At higher IgG concentrations, the agglutinates were very difficult to dissociate by vortexing. This was reflected in the cell-cell adhesion assay, in that the number of single cells was very low before gyration was begun (Fig. 2). However, when the cell-cell adhesion assay was performed by gyration in the presence of GARFab, there was a progressive inhibition of cell-cell adhesion as a function of the concentration of anti-*Dd* with which the cells were initially incubated over a range of relatively low IgG concentrations (Fig. 2). Inhibition was observed whether or not the assays were performed in the presence of EDTA, indicating that the anti-*Dd* IgG contained components that could block the distinct types of cell-cell adhesion observed under these two conditions (2, 9). Under these assay conditions and at very high IgG concentrations, agglutination partially obscured the inhibition of cell-cell adhesion (Fig. 2).

The relationship between time of gyration and the concentration of inhibitory antibody bound to the cells was explored in some detail to determine an optimum incubation time for a wide range of antibody concentrations. One example of these studies is shown in Fig. 3. Here we show that with high concentrations of anti-*Dd* IgG, which produce extensive initial agglutination, complete inhibition of cell-cell adhesion was ultimately obtained after prolonged gyration in the presence of an excess of GARFab. In contrast with lower amounts of IgG, marked inhibition was seen much earlier and was actually diminished with more prolonged gyration, presumably because

¹ Springer, W. R., P. L. Haywood, and S. H. Barondes. *J. Cell Biol.* 87: 682-690.

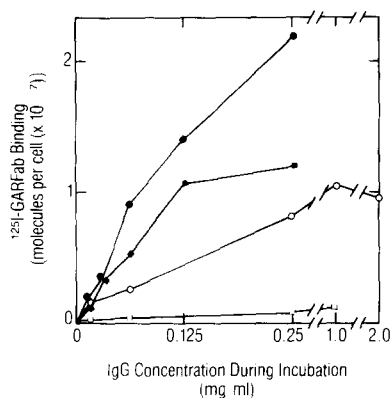


FIGURE 1 ^{125}I -GARFab binding to cohesive *D. discoideum* cells after preincubation for 30 min at 4°C with a series of concentrations of four rabbit IgGs. ^{125}I -GARFab binding to washed cells was determined, as given in Materials and Methods, after incubation with the indicated concentrations of the following IgGs: anti-*Dd*, ●; anti-GP150, ■; antidiscoidin, ○; normal IgG, □.

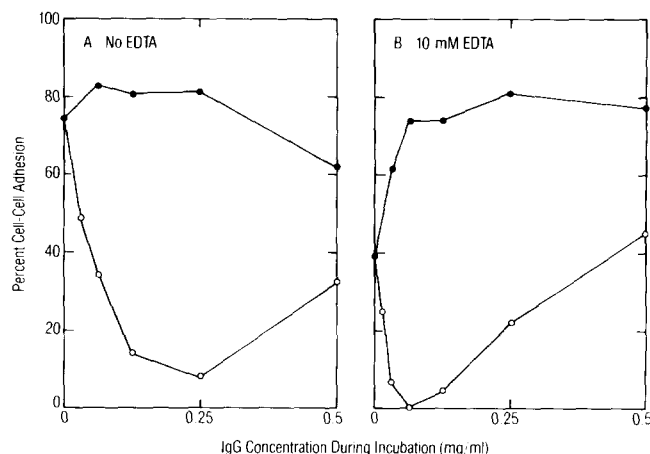


FIGURE 2 Cell-cell adhesion assays performed in the presence (○) or absence (●) of GARFab after preincubation with anti-*Dd* rabbit IgG. Cells were preincubated with the indicated amounts of anti-*Dd* IgG. Cell-cell adhesion was determined by electronic particle counting after gyration in a solution with or without GARFab (final concentration 0.5 mg/ml) as described in Materials and Methods. A, assay without EDTA; B, assay with 10 mM EDTA.

of progressive release of sufficient anti-*Dd* IgG from the cell surface to fall below a critical inhibitory concentration.

The two cases in Fig. 3 illustrate both the inherent limitations of the cell-cell adhesion assay in evaluating antibodies directed against cell adhesion molecules and also the fact that they can be overcome by proper choice of conditions. One limitation is that any bound anti-*Dd* IgG that spontaneously dissociated from the cell surface during the cell-cell adhesion assay would be trapped in the incubation medium by the excess of GARFab. Because it would be irreversibly lost to the cell surface, its inhibitory activity would be curtailed. We believe that this explains the progressive loss of inhibitory activity found with the low concentration of anti-*Dd* IgG in Fig. 3. The other limitation is that high concentrations of anti-*Dd* IgG produce cellular agglutination. Presumably, at these high concentrations, enough anti-*Dd* IgG is bound to the surface in a configuration where one valence is bound to an antigen on each of two adjacent cell surfaces, leading to agglutination. When one

or the other of these bonds dissociates spontaneously in the presence of an excess of GARFab, this portion of the IgG would be expected to be bound by the GARFab and prevented from reassociating with another cell. One would, therefore, expect a time-dependent diminution in agglutination, thereby allow for detection of the inhibitory effect on cell-cell adhesion. This is how we interpret the results with the high concentration of anti-*Dd* shown in Fig. 3. We recognize that operationally agglutination and adhesion cannot be distinguished by the electronic particle counter. One way we know that we are dealing with agglutination at the high IgG concentration is that we find large clumps which are never seen under identical gyration conditions in the absence of antibody. Another indication is that the clumps of cells break up when gyrated in GARFab (Fig. 3) but not in its absence.

One major conclusion from the types of experiments shown in Fig. 3 is that it is critical that appropriate experimental conditions be defined in screening for potential antibodies against cell adhesion molecules. In practice, in the studies with slime molds, we found that 10–20 min of gyration in the cell-cell adhesion assay was adequate to detect inhibitory actions of a wide range of concentrations of antibodies directed against putative cell adhesion molecules. In other studies with much lower concentrations of anti-*Dd* IgG, we found marked inhibition of cell-cell adhesion with 10-min incubations but much smaller effects with 20-min incubations. With small amounts of antibody, as in screening studies with monoclonal antibodies, the shorter incubation would be expected to be a more sensitive condition. Were this approach applied to other types of cellular systems, appropriate conditions would have to be determined. It remains possible that some antibodies against cell-cell adhesion molecules might not be detected by these screening conditions. However, this limitation is shared by other types of screening procedures, such as the use of univalent fragments of primary antibodies. In that case, the lower affinity of the univalent fragments and the limited amount of available antibody might be disabling.

We also evaluated the amount of GARFab that was necessary in these assays. We found that when *D. discoideum* cells were incubated with concentrations of anti-*Dd* IgG as high as 0.25 mg/ml, maximum inhibitory effects could be produced when the second incubation was performed in a concentration

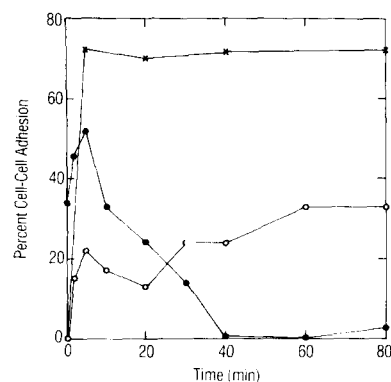


FIGURE 3 Time-course of inhibition of cell-cell adhesion at two anti-*Dd* IgG concentrations. Cells were preincubated with 0.05 mg/ml (○) or 0.5 mg/ml (●) of anti-*Dd* IgG or 0.5 mg/ml normal rabbit IgG (×), then mixed with 11 vol of a solution containing GARFab (final concentration 0.5 mg/ml) but no EDTA. The mixture was gyrated for the indicated periods at which time the percent cell-cell adhesion was determined with an electronic particle counter.

of GARFab as low as 0.1 mg/ml. In practice, we kept the GARFab concentration at 0.5 mg/ml, a large excess.

Having established these conditions with anti-*Dd* IgG, we studied the effects of a more specific reagent, anti-GP150 IgG, at various concentrations (Fig. 4). At high concentrations, there was significant cell agglutination, as indicated at the zero time-points. The inhibitory activity of anti-GP150 IgG on cell-cell adhesion could, however, be clearly demonstrated with the GARFab technique. The amount of anti-GP150 and anti-*Dd* IgG required to inhibit cell-cell adhesion by 50% in this assay was quite low (Table I); and the concurrent ^{125}I -GARFab binding at that inhibitory concentration was similar for the two IgGs (Table I).

The finding that binding equivalent amounts of IgG and GARFab directed against a mixture of antigens on the one hand and a specific antigen on the other produced similar effects on cell-cell adhesion raised the question that cell-cell adhesion might be blocked by binding a comparable amount of a rabbit immunoglobulin raised against any cell surface antigen followed by the binding of an equivalent amount of GARFab. This possibility was ruled out by the finding that antidiscoidin IgG binding to the cell surface over a wide range of concentrations (as shown by ^{125}I -GARFab binding in Fig. 1) had very little inhibitory effect on cell-cell adhesion of differentiated *D. discoideum* cells (Fig. 5). Univalent antibody fragments made from antidiscoidin IgG also failed to substantially influence cell-cell adhesion in concentrations up to 5 mg/ml (data not shown). Although considerable evidence has been presented that discoidin plays a role in cell-cell adhesion in *D. discoideum* (1, 11), that role is not apparently reflected in the assay of cell-cell adhesion used in the present experiments. Except under unusual assay conditions, we (12) and others (13) have failed to inhibit cell-cell adhesion of another species of slime mold with univalent antibody fragments directed against its cell surface lectin. Whatever the significance of these results, they support the major point of this paper, the consistency of results with the present technique and with univalent antibody fragments made from the primary antibody. It is also notable that, despite marked binding of antidiscoidin IgG to the cell surface, we found very little agglutination of the cells, in striking contrast with the effects of anti-*Dd* IgG and anti-

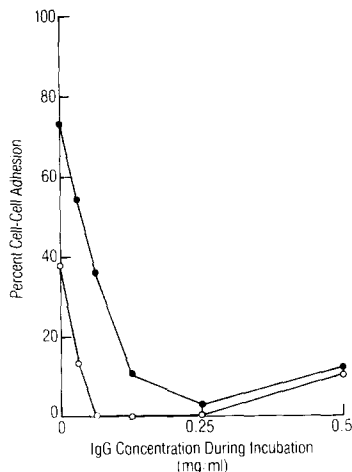


FIGURE 4 Inhibition of cell-cell adhesion after incubation with anti-GP150 IgG followed by GARFab. Cell-cell adhesion assays with (O) or without EDTA (●) were performed by gyration in GARFab after preincubation with the indicated concentrations of anti-GP150 IgG.

TABLE I

Concentration of IgGs Needed to Inhibit Cell-Cell Adhesion (Assayed with or without EDTA) by 50%; and Amount of This IgG Bound to the Cell Surface as Inferred by ^{125}I -GARFab Binding

IgG	EDTA mM	Rabbit IgG added that inhibited cell-cell adhesion by 50%	
		(mg/ml)	^{125}I -GARFab molecules bound/cell $\times 10^{-6}$
Anti- <i>Dd</i>	10	0.02	2.5
	0	0.08	10.3
Anti GP150	10	0.02	1.5
	0	0.06	6.7

Aggregating *D. discoideum* cells were suspended and incubated with a series of concentrations of IgG as described in Materials and Methods. Aliquots were then removed and gyrotated with 0.5 mg/ml of GARFab in an incubation mixture with or without 10 mM EDTA for 20 min, and cell-cell adhesion was determined with an electronic particle counter. The concentration of IgG that inhibited cell-cell adhesion by 50% is indicated. Another aliquot of cells was centrifuged through Ficoll, incubated with ^{125}I -GARFab, and the amount of ^{125}I -GARFab bound/cell, a measure of the amount of rabbit antibody that had been bound, was determined as described in Materials and Methods.

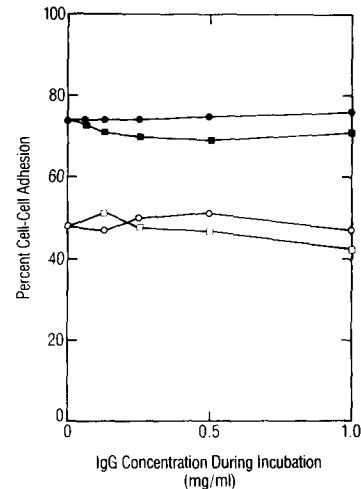


FIGURE 5 Effect of GARFab on cell-cell adhesion measured with or without EDTA, with antidiscoidin IgG or normal IgG. Conditions were: normal IgG assayed without EDTA (●); antidiscoidin IgG assayed without EDTA (■); normal IgG with EDTA (O); antidiscoidin IgG with EDTA (□).

GP150 IgG. The significance of this result remains to be determined.

DISCUSSION

The above results indicate that it is possible to identify antibodies against putative cell adhesion molecules in slime molds by using a univalent second antibody to prevent agglutination mediated by the primary antibody. For use in large scale screening procedures, this assay has the distinct advantage of not requiring preparation of univalent antibodies from the specific sera. Indeed, we have found that immune serum diluted fivefold or more can be used in place of the IgG during the preincubation with identical results to those shown above (data not shown). Likewise, dilution of IgG into the conventional medium used for raising monoclonal antibodies (6) yields

identical results. Therefore, it is possible to directly screen these products without further preparation.

Inhibition by this indirect procedure also requires less antibody than procedures that use univalent fragments of the primary antibody. For instance, Geltosky et al. (4) required 0.6 mg/ml anti-GP150 Fab' to get complete inhibition of cell-cell adhesion in their assay system. A similar concentration of Fab was used by Müller and Gerisch (3) in their identification of "contact sites A." In contrast, under similar assay conditions we required only ~3% as much antibody (by weight) and only ~1% the concentration of IgG molecules compared with the concentration of univalent antibody molecules used by others. Although these comparisons are not direct, we did use the same IgG preparation used by Geltosky et al. (4); and the fact that we required a similar IgG concentration under two very different assay conditions (Table I) increases the plausibility of comparison with work in other laboratories that did not use identical experimental conditions. The increased sensitivity of the present approach is probably because of the higher affinity of IgG for antigens when compared with its univalent fragments. This increased sensitivity also makes such an assay practical for screening hybridoma supernates for activity. As seen from Table I, only 20–60 µg/ml of anti-GP150 IgG is needed to inhibit cell-cell adhesion by 50%. Because it is unlikely that >10% of this mixed IgG is directed against GP150, concentrations of IgG in the range of a few micrograms per milliliter should be detectable as inhibitors of cell-cell adhesion. Screening of the antibody products of initial cultures of hybridomas should, therefore, be feasible. Although this work indicates that the univalent second antibody technique studied here gives results consistent with those found using a univalent primary antibody, it is important to recognize that this entire approach is limited by the inherent difficulties of

choosing meaningful in vitro assays of cell-cell adhesions. These limitations, and the importance of simultaneous alternative approaches to this problem, are considered in detail elsewhere (1).

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