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Down's syndrome lymphoid cell lines exhibit increased adhesion due to the over-expression of lymphocyte function-associated antigen (LFA-1)

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SUMMARY

We have analysed homotypic adhesion induced by the phorbol ester TPA in EBV-immortalized Down's syndrome (trisomy 21) and normal lymphoblastoid cell lines. Our results show that the trisomy 21 cells aggregated more readily than normal cells. The aggregation of the two types of cell was blocked effectively by the addition of monoclonal antibodies to the α and β chains (CD11a, CD18) of lymphocyte function-associated antigen (LFA-), but not by monoclonal antibodies to LFA-3 and the α chain of p150,95 (CD11c). Since TPA did not increase the expression of CD18 on trisomy 21 cells, we conclude that the increased adhesiveness is the result of over-expression of CD18 as a result of gene dosage.

INTRODUCTION

Down's syndrome is a constitutional trisomic condition resulting from duplication of chromosome 21 (Epstein, 1986). It has an overall frequency of 1/600–1/700 live births, but a markedly increased incidence in relation to maternal age at conception (Smith & Warren, 1985).

There is a high level of post-natal mortality in Down's syndrome due in part to a raised incidence of infectious disease (Thase, 1982). Overall mortality in Down's syndrome by the age of 30 years is estimated to be as high as 50%. Despite extensive studies directed at finding an underlying cause of the impaired immune response in Down's syndrome (reviewed by Epstein, 1986), no single factor has yet been identified.

Our studies have established that trisomy 21 lymphoid cells express more lymphocyte function-associated antigen (LFA-1) than normal (Taylor, Williams & D'Souza, 1986; Taylor *et al.*, 1987, 1988). LFA-1 is a heterodimeric cell membrane molecule in which a 95,000 MW β chain (CD18) is non-covalently associated with a 180,000 MW α chain (CD11a) in the form of α/β complexes (Sanchez-Madrid *et al.*, 1983). LFA-1 behaves as a general cell–cell adhesion molecule between lymphoid and myeloid cells (Springer *et al.*, 1984; Hynes, 1987) and promotes the efficiency of T-cell reactions such as cytotoxic T-cell–target binding (Dongworth *et al.*, 1985). The β chain of LFA-1, CD18, is common to two other adhesion molecules, OKM1 and p150,95 which have distinct α chains, CD11b and CD11c, respectively (Sanchez-Madrid *et al.*, 1983; Cobbold, Hale & Waldmann, 1987) and more restricted distributions and functions than LFA-1.

The increased expression of LFA-1 on trisomy 21 cells can be attributed to the effects of gene dosage, since the CD18 gene is located on chromosome 21 (Suomalainen *et al.*, 1986; Marlin *et al.*, 1986). CD18 is necessary for the expression of CD11a (Springer *et al.*, 1984), and formation of CD18/CD11a complexes on the surface of trisomy 21 cells may be up-regulated by an over-production of CD18.

Recent studies using Epstein–Barr virus (EBV) immortalized lymphoid cells have demonstrated that LFA-1 is important in homotypic intercellular adhesion (Patarroyo *et al.*, 1986; Rothlein & Springer, 1986). In view of these findings we postulated that increased expression of LFA-1 by trisomy 21 cells might also cause an increase in the aggregation of trisomy 21 cells. The results presented in this paper show that this indeed is the case.

MATERIALS AND METHODS

Cell lines

Lymphocytes obtained from people with Down's syndrome and from normal controls were immortalized by infection *in vitro* with Epstein–Barr virus containing culture medium derived from the B95-8 marmoset cell line, as described previously (Taylor *et al.*, 1988). Lymphoblastoid cell lines (LCL) were routinely grown in RPMI-1640, containing 10% fetal calf serum (FCS), 10% tryptose phosphate and antibiotics (Northumberland Biological, Cramlington, Northumberland), and used only when negative for mycoplasma.

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Monoclonal antibodies

Monoclonal antibodies (mAb) to subunits of the leucocyte adhesion complex, CD11a CD11c, CD18, were obtained as gifts form Dr Patrick Beatty, Fred Hutchinson Cancer Research Center, Seattle, Washington, DC and Professor Andrew McMichael, Nuffield Department of Clinical Medicine, University of Oxford (60.3; MHM23, MHM24, respectively), and through the *Third Leucocyte Differentiation Antigen Workshop* (McMichael *et al.*, 1987). The anti-HLA class I mAb W6/32 was obtained from Sera-lab (Crawley, Sussex). They were used at dilutions indicated in the text.

Immunofluorescence and flow cytometry

Analysis of antigen expression by cells with or without TPA treatment was carried out by indirect membrane immunofluorescence and flow cytometry, as previously described (Taylor *et al.*, 1988). Cells were incubated first at 1×10^6/ml in culture tubes for 30 min in the presence of 5 ng/ml TPA, then washed in phosphate-buffered saline (PBS; Oxoid, Basingstoke, Hants) containing 0·1% bovine serum albumin (BSA; Sigma, Poole, Dorset). The cells were then incubated sequentially with excess monoclonal antibody and goat anti-mouse Ig-FITC and resuspended in PBS–BSA for analysis. This was carried out on a FACS IV (Becton-Dickinson, Sunnyvale, Ca, USA) at a PMT of 700 V, and laser settings of 200 mW at a wavelength of 488 nM. Twenty-thousand cells were analysed and the results plotted as profiles on linear axes.

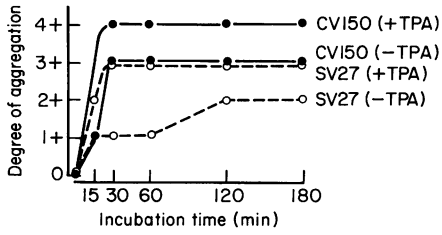

Figure 1. Effect of incubation time on the aggregation of trisomy 21 (CV105) and normal (SV27) EBV-LCL, in the presence or absence of 5 ng/ml TPA. The degree of aggregation was scored as described in the Materials and Methods.

Table 1. Aggregation of normal and trisomy 21 LCL

	TPA conc. (ng/ml)*		
Cell line	0	1·0	10
Normal			
SV20	1+†	1+	2+
SV26	1+	1+	1+
SV27	1+	1+	3+
SV29	1+	2+	2+
Trisomy 21			
CV77	0	1+	0
CV79	2+	2+	4+
CV105	2+	4+	4+
CV108	2+	2+	2+

* Final concentration of TPA.

† Aggregate scores as described in the Materials and Methods.

Aggregation assay

Homotypic adhesion was measured by the semi-quantitative method described by Rothlein & Springer (1986), with modifications. The LCL were washed and resuspended in RPMI-1640 containing 1 mM HEPES buffer (Flow Labs, Rickmansworth, Herts) and 5% FCS. Aliquots of 90 μl, containing 4×10^5 cells, were dispensed into 96-well flat-bottomed plates and 10 μl of an appropriate concentration of 12-0-tetradecanoyl phorbol-13 acetate (TPA, Sigma) were added. Control wells containing cells but not TPA were set up at the same time. The plates were usually incubated for 30 min at 37° in a CO_2 incubator. The degree of aggregation as an index of adhesion was scored by examining the wells under an Olympus inverted microscope. Clusters of aggregated cells were scored thus: 0, none; 1+, < 10%, 2+, 10–50%; 3+, > 50% of cells in small clusters; 4+, 5+ virtually 100% of cells in large compact clusters. All wells were scored in duplicate and in most cases checked by two observers. For studies on the inhibition of adhesion by mAb, duplicate wells of each cell with or without monoclonal

Table 2. Effect of mAb* on aggregation of LCL

		Aggregation of:†					
		Normal			Trisomy 21		
mAb‡	Specificity	SV27	SV28	SV29	CV86	CV105	CV108
Experiment 1							
Control§	—	2+	2+	2+	4+	4+	2+
60.3	CD18	0	0	0	0	0	0
MHM23	CD18	0	0	0	0	0	0
CLB.54	CD18	0	0	0	0	0	0
M232	CD18	0	0	0	0	0	0
MHM24	CD11a	0	0	0	0	0	0
C1MT	CD11a	2+	1+	3+	4+	4+	1+
25.3.1	CD11a	0	0	0	0	0	0
25.5.2	CD11a	0	0	0	0	0	0
2F12	CD11a	0	0	0	0	0	0
Leo LFA-1	CD11a?	0	2+	0	0	0	0
3.9	CD11a	3+	2+	4+	5+	5+	4+
Experiment 2							
Control	—	3+	2+	3+	4+	4+	2+
TS1/18	CD18	2+	0	3+	2+	2+	2+
1B4	CD18	1+	0	0	0	0	0
TS1/12	CD11a (1)	1+	0	0	0	0	0
TS1/22	CD11a (3)	3+	0	3+	0	1+	1+
TS2/6	CD11a (2)	0	0	0	0	1+	1+
CLB LFA1/2	CD11a	2+	0	1+	0	1+	1+
3.9	CD11c	3+	1+	3+	5+	4+	4+
L29	CD11c	2+	2+	3+	5+	4+	4+
3KB43	CD11c	2+	2+	3+	5+	4+	4+
KB23	CD11c	2+	2+	3+	5+	4+	4+
SHCL-3	CD11c	3+	2+	3+	5+	5+	5+
TS2/9	LFA-3	4+	3+	4+	5+	5+	5+

* MAb obtained through third Leucocyte Differentiation Antigen Workshop are nos. 706–730 of the 'CD18' panel.

† Aggregation scored as described in the Materials and Methods.

‡ MAb used at final concentration of 1/100 from stock.

§ Control wells contained cells + TPA, but not mAb.

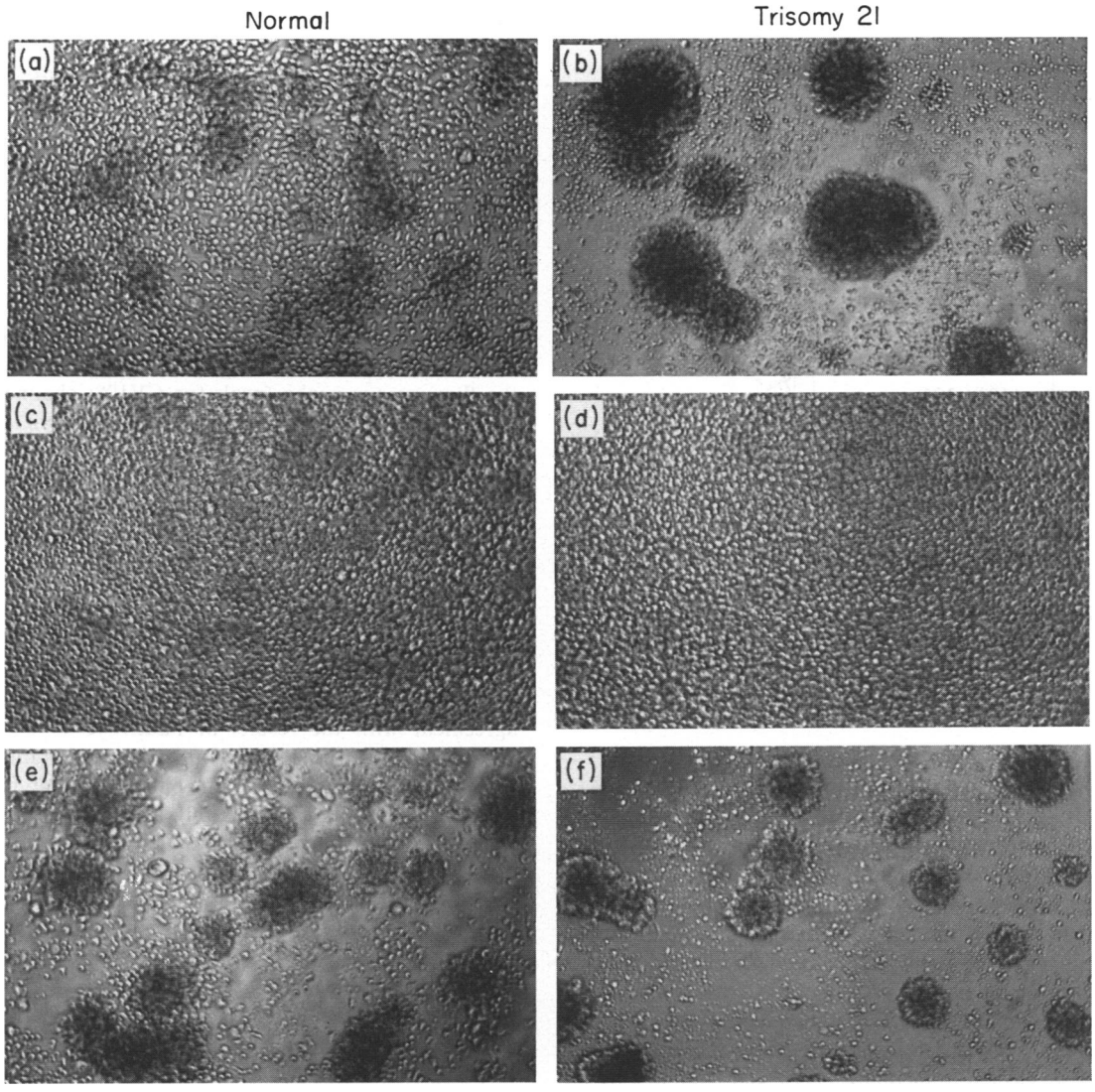

Figure 2. Effect on aggregation of incubating a normal (SV28; a,c,e) and trisomy 21 (CV86; b,d,f) EBV-LCL for 30 min in the presence of 5 ng/ml TPA, and no mAb (a,b), with the CD18 mAb 60.3 (c,d) or the LFA-3 mAb TS2/9 (e,f).

antibody were set up. mAb were initially diluted from stock at 1/20, and added to aggregation assays to give a further dilution of 1/5 and 1/10.

RESULTS

Preliminary studies were carried out to determine which incubation conditions would reveal differences in adhesion by trisomy 21 compared with normal LCL. In these experiments it became clear that the transfer of cells after incubation in 96-well plates to a haemocytometer to count the clusters caused them to break up. In addition, the time required to count each sample added significantly to the overall incubation time, and precluded comparison of more than one pair of LCL (trisomy 21 versus normal) in each experiment.

For these reasons we adopted the 'qualitative' aggregate scoring assay (see the Materials and Methods) of Rothlein & Springer (1986). Immediately after incubation the 96-well plate was examined under an inverted microscope, and an aggregate score of 0–9+ assigned, by reading duplicate wells of each cell. This allowed us to read as many as eight LCL in each experiment, or to compare the effect of different incubation conditions and monoclonal antibodies on adhesion.

Initially, experiments were carried out in which trisomy 21 and normal cells were incubated for different times (15 min–3 hr) at various cell concentrations ($1–4 \times 10^6$/ml) and in the presence of a range of TPA concentrations (1,5, and 10 ng/ml). In Fig. 1 the aggregation of a trisomy 21 (CV105) and normal LCL (SV27) in the presence or absence of 5 ng/ml TPA is compared over a 3 hr incubation time. Adhesion of both cells occurred readily within 15 min and was complete by 30 min, but the trisomy 21 cells formed larger aggregates than the normal cells, and in both cases TPA increased the size and number of the cells involved in the clusters.

Table 1 compares adhesion by four trisomy 21 (CV77, CV79, CV105, CV108) and four normal LCL (SV20, SV26,

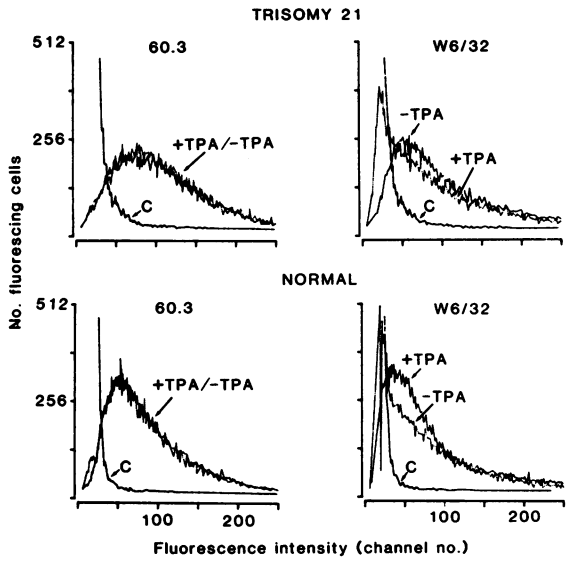

Figure 3. Flow cytometric analysis of CD18 and HLA class I expression by trisomy 21 (CV108) and normal (SV29) EBV-LCL, detected by the mAb 60.3 and W6/32, respectively, on TPA and untreated cells. Cells were stained indirectly with anti-mouse Ig-FITC; C is the conjugate-only control. Profiles are drawn on linear axes.

SV27, SV29) incubated for 30 min in 10, 1 (ng/ml) or no TPA. Three of the four trisomy 21 cells formed larger aggregates than three of the four normal cells in 1 ng/ml TPA. With higher concentrations of TPA such as 10 ng/ml, the difference was not as marked, largely because normal cells aggregated more strongly. Although the trisomy 21 cells tended to aggregate in the absence of TPA, the results were less consistent than when it was present in the medium.

One of the trisomy 21 cells, CV77, aggregated poorly even in the presence of TPA (see Discussion). From these and other experiments we elected to incubate 4×10^5 cells/well in 5 ng/ml TPA for 30 min. Studies on the requirement for FCS for cluster formation showed that 2/3 normal cells but 0/3 trisomy 21 cells formed aggregates in the absence of FCS. No advantage accrued from agitating the plates during incubation.

The role of LFA-1 in the adhesion of trisomy 21 cells was investigated by adding CD11a and CD18 monoclonal antibodies TPA-induced LCL. Table 2 shows the results obtained with three trisomy 21 and three normal cell lines tested in the same experiments with a series of CD18 and CD11a mAb. As controls the cells were incubated with mAb to the α subunit of p150,95 (CD11c), which is poorly expressed by LCL, and with the LFA-3 mAb TS2/9, which reacts strongly with normal and trisomy 21 LCL (data not shown). With the exception of TS1/18 all of the CD18 mAb markedly inhibited aggregation of both the normal and trisomy 21 LCL. The CD11a mAb were more variable in their inhibitory activity. Neither the CD11c nor the LFA-3 mAb had any inhibitory effect on aggregation.

Results illustrating the differences in aggregation of a normal and trisomy 21 cell, and the effect of incubation with the CD18 mAb 60.3, and the LFA-3 mAb TS2/9 are shown in Fig. 2. The trisomy 21 (CV86) LCL clearly formed larger clusters than a normal (SV28) LCL, but these were inhibited by the CD18 mAb 60.3. In the presence of anti-LFA-3, the trisomy 21 clusters are smaller but were not inhibited. An unexpected finding was that titration of certain CD11c mAb slightly increased the size of aggregates obtained with certain cells, though the reasons for this are not clear.

Increased aggregation by the trisomy 21 cells may have been due to altered CD18 expression in response to TPA treatment. Flow cytometric analysis was therefore used to compare the reactivity of trisomy 21 (CV108) and normal LCL (SV29) with 60.3 (anti-CD18) following incubation with or without TPA. The FACS profiles in Figure 3 show that TPA had no effect on the expression of CD18 by the two types of LCL.

However, there is a difference in the CD18 profiles of the normal and trisomy 21 cell, as shown clearly by the position of the fluorescence peaks (peak for CV108: channel 70; SV29: channel 49, ratio 1·42 at a fluorescence gain of 8). In contrast to the lack of effect on CD18, TPA treatment of the trisomy 21 cells slightly reduced HLA class I (W6/32) expression, but increased it on normal cells. These results were confirmed with two different trisomy 21 and normal cell lines (not shown).

DISCUSSION

Our results demonstrate for the first time that Epstein–Barr virus immortalized trisomy 21 lymphoblastoid cell lines exhibit increased homotypic adhesion compared with normal cells. In agreement with the results of Patarroyo *et al.* (1986) and Rothlein & Springer (1986), homotypic cellular adhesion by the EBV-LCL was promoted by the phorbol diester TPA. At high TPA concentrations (10 ng/ml) and prolonged incubation (>1 hr) the trisomy 21 and normal LCL formed similar aggregates. However, by incubating the cells for 30 min in 5 ng/ml TPA, the difference in the size of aggregates could be seen more clearly. With one exception, all trisomy 21 cells formed larger and more compact clusters than normal cells, similar to the results shown in Fig. 2.

Some of the trisomy 21 cells formed aggregates in the absence of TPA, but here the differences with normal cells were less easy to quantify. One trisomy 21 cell line (CV77) did not form aggregates easily, even after TPA treatment. This LCL was derived from lymphocytes of a patient of West African origin and grows more aggressively than most trisomy 21 EBV-LCL. The lack of spontaneous clusters of CV77, despite the expression of more CD18 than on normal cells, is intriguing and suggests that expression of the LFA-1 ligand may be lacking. Extensive phenotypic analysis of these cells revealed that they are CD1–8 negative, indicating that they are not T cells. They are also negative for CD9 (p24), and CD19, but positive for CD23, a restricted B-cell activation antigen (Ling, MacLennan & Mason, 1987). Expression of cell-surface IgM is consistent with a B-cell phenotype, but it is not clear whether these cells have acquired a malignant phenotype.

Addition of CD18 and CD11a monoclonal antibodies to TPA-stimulated trisomy 21 and normal LCL in most cases completely inhibited aggregation, confirming that both subunits of the LFA-1 molecule are involved in adhesion (Rothlein & Springer, 1986). We conclude from this evidence that the increase in the adhesive capacity of trisomy 21 cells is related to greater expression of LFA-1 compared with normal cells. Previously we have shown that the expression of both CD11a and CD18 is increased on trisomy 21 compared with normal cells (Taylor *et al.*, 1986, 1987, 1988), probably as a result of

duplication of the CD18 gene, which is coded by chromosome 21 (Suomalainen *et al.*, 1986; Marlin *et al.*, 1986). The increased CD11a expression by trisomy 21 cells is most probably due to the regulatory effect of excess CD18 chains on CD11a expression, since Springer *et al.* (1984) have shown that cells lacking CD18 are CD11a negative.

The monoclonal antibody inhibition studies suggest that both CD18 and CD11a are involved in the increased homotypic adhesion observed with trisomy 21 cells. That inhibition is due to the blocking of a cell surface molecule is indicated by the lack of inhibitory effect by mAb to CD11c, which is absent from EBV-LCL. Moreover adhesion was not inhibited by the LFA-3 mAb TS2/9. This 60,000 MW polypeptide (Sanchez-Madrid *et al.*, 1982) does not appear to be involved in LFA-1-mediated adhesion, but acts as the ligand for the T-cell associated CD2 molecule (Selvaraj *et al.*, 1987).

With the exception of LFA-3 we cannot exclude the contribution of other molecules in homotypic cellular adhesion by trisomy 21 LCL cells. However, other workers have reported that mAb to a spectrum of non-LFA-1 leucocyte cell surface antigens had no effect on homotypic adhesion (Rothlein & Springer, 1986; Patarroyo *et al.*, 1986). The result showing a lack of aggregation by the trisomy 21 LCL CV77 suggests that these cells may lack the ligand for LFA-1. Recent evidence (Makgoba *et al.*, 1988) has shown that an intercellular adhesion molecule (ICAM-1) expressed in several glycosylated forms by leucocytes and non-leucocytes (Dustin *et al.*, 1986) is the ligand for LFA-1.

There was no evidence that TPA itself caused an increase in the expression of CD18 on trisomy 21 cells, suggesting that its functional effect in promoting adhesion is similar to that on normal cells. The small change in HLA class I expression caused by TPA is similar to that reported by Rothlein & Springer (1986). TPA is a tumour promotor with a diacylglycerol-like structure, able to replace the latter by directly activating protein kinase *C* (Nishizuka, 1984). Protein-kinase *C* plays an important role, in the presence of $Ca2^+$ in signal transduction into cells. It is thus significant that adhesion mediated by LFA-1 is both TPA and divalent cation ($Ca\ 2^+$, $Mg2^+$) dependent (Rothlein & Springer, 1986). Whether there is increased protein kinase *C* synthesis by resting and TPA-activated trisomy 21 cells has not yet been demonstrated. However, increased aggregation of the trisomy 21 LCL could be due simply to the quantitative effect of LFA-1 on the binding of the LFA-1 ligand ICAM-1, in the presence of $Ca2^+$ and protein kinase *C*.

Intercellular co-operation between lymphoid cells is a necessary requirement for optimum immune responses. These effects are now known to involve accessory receptor–ligand interactions as well as the recognition of specific antigens by their V-gene coded receptors. LFA-1 is one such accessory molecule that may assist low-affinity intercellular contacts, such as those between T-cell receptors and antigen. In Down's syndrome however, excessive homotypic adhesion could adversely affect leucocyte interactions by preventing cytotoxic T cells adhering specifically to virally infected target cells.

Previous studies showing that lung fibroblasts from Down's syndrome exhibit increased adhesive capacity (Wright *et al.*, 1984) attributed this to the effect of gene dosage on the expression of an unidentified cell-surface molecule coded by chromosome 21. Though adhesion was divalent cation dependent, it is unlikely to be due to LFA-1, since CD11a/CD18 expression is restricted to lymphoid cells. However, it is possible that a related molecule, perhaps another member of the integrin family (Hynes, 1987) expressed by non-lymphoid cells could be involved.

Since we were able to distinguish, with one exception, trisomy 21 from normal cells by their adhesive capacity, it is possible that this could be used as the basis of a diagnostic test for Down's syndrome. For antenatal diagnosis, this would require the sampling of fetal blood *in utero*, normally a difficult and hazardous procedure. A more immediate clinical application would be in the confirmation of diagnosis in the immediate post-natal period and an assessment of the possible consequences of increased adhesion on immune functions in Down's syndrome. Further research should enable this to be more fully evaluated.

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