The Contribution of Assays for Lymphocyte Capping and Creatine Kinase to Detection of the Becker-Type Dystrophy Trait

Barbara M. Goldsmith, Hanns-Dieter Gruemer, Rollin J. Hawley, Nathan A. Pickard, Harland L. Verrill, Walter E. Nance, Gregory Miller, and Ralph G. Crawford

Members of three unrelated families with the mild Becker type of muscular dystrophy were subjected to lymphocyte capping tests and measurements of serum creatine kinase activity. Both tests correctly identified all nine affected males, but only the capping test was abnormal in seven of eight obligate carriers. The number of capped cells in carriers and affected persons with the Becker-type dystrophy was generally intermediate between those observed for individuals with the Duchenne trait and normal controls, thus potentially aiding in the differential diagnosis between the two myopathies. The lack of sensitivity of measurements of serum creatine kinase activity in identifying carriers is further complicated by the difficulty of establishing reliable reference intervals for this enzyme in 204 healthy controls. Detailed directions for the performance of the capping test are presented.

Additional Keyphrases: heritable disorders - benign X-linked muscular dystrophy - diagnostic aids - carrier detection - degenerative myopathy - genetics - Becker-type muscular dystrophy

Of the inherited degenerative myopathies, sex-linked Duchenne’s muscular dystrophy is the most common form; its estimated incidence is between 2.5 and 1.6 per 10 000 (1). Characteristically, symptoms of the disease are manifested early in life. Initially, there is weakness in the pelvic-girdle region; progression is rapid, with significant wasting of other proximal muscle groups within several years. Afflicted boys are usually wheelchair-bound by the age of 10 years, and usually die during the late teens, rarely surviving into the third decade.

In 1955, Becker and Kiener (2) separated from this disease a less frequent variant of X-linked dystrophy; numerous additional case descriptions of benign sex-linked muscular dystrophy appeared in rapid succession (3-7), and the incidence of this form of dystrophy is now estimated to be 1.8 per 100 000. Data for gene linkage of the two types of muscular dystrophy to color blindness, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) variants, and Xg blood groups also suggest that the two dystrophies are distinct genetic entities (8-10).

Muscular dystrophy of the Duchenne and Becker types are clinically rather similar, and the differential diagnosis is based primarily on the rate of progression. According to Emery and Skinner (7), the best discriminating criterion is the age at which the patient becomes chair-bound: in Becker dystrophy the mean age is 27 years, nearly 20 years later than in the Duchenne-type dystrophy. Thus, any afflicted boy with a biopsy-confirmed degenerative myopathy and able to walk beyond the age of 12 years should be evaluated for Becker- or limb-girdle-type muscular dystrophy, depending on the clinical findings and relevant family history.

We previously reported the occurrence of diminished lymphocyte capping (lateral movement of cell-surface proteins and their aggregation into a cap) in 25 families with Duchenne-type dystrophy and four in one family with Becker-type dystrophy (11, 12). Since then we have had the opportunity to observe two additional families with the benign sex-linked muscular dystrophy (1). Because men with Becker dystrophy are fertile, unlike patients with the rapidly progressing form, we wanted to determine whether the capping test could correctly identify obligate carriers and to compare the results by capping with the values for creatine kinase activity in serum or plasma.

Materials and Methods

Reagents

Seligmann’s balanced salt solution (SBSS) was prepared by dissolving 61.2 g of NaCl, 1.6 g of KCl, 12.0 g of sodium acetate, 0.4 g of NaH2PO4 · H2O, 0.8 g of KH2PO4, 3.6 g of NaHCO3, 8.0 g of dextrose, 24 mg of ascorbic acid, and 4.0 g of (ethylenedinitriilo)tetraacetic acid in distilled water and diluting to 2 L after adjusting the pH to 6.7 with NaOH. Before use, we diluted this stock solution fourfold with distilled water; the pH was then 7.2, and the osmolality was between 295 and 305 mosM/L.

Ficoll-Paque for the isolation of mononuclear cells (Pharmacia Fine Chemicals, Piscataway, NJ 08854).

Goat-derived fluorescein isothiocyanate (FITC) conjugated “polyvalent” anti-human immunoglobulins (IgG, IgA, IgM): high fluorescein:protein (F/P) ratio (Kent Lab., Redmond, WA 98052).

Procedures

Isolation of human mononuclear leukocytes. Heparinized blood (15 mL) was collected and kept at room temperature. Within 4 h the plasma was separated by centrifugation (10 min, 1100 × g, 25 °C) for later measurements of enzyme activity. The cells were resuspended in about 30 mL of SBSS and transferred into a 50-mL polycarbonate tube. We layered 10 mL of the Ficoll-Paque beneath the cell suspension by use of a syringe fitted with a small-bore metal tube to which a piece of polyethylene tubing was connected. Centrifugation of the samples (500 × g, 25 min, 25 °C) resulted in the following four layers (bottom to top): erythrocytes, Ficoll-Paque, a thin layer containing lymphocytes and other mononuclear leukocytes, and SBSS.

We removed the lymphocyte-containing layer, using the same syringe attachment described above. We collected about 10 mL of lymphocytes and SBSS by skimming the leukocyte layer, taking care not to stir the cells or to obtain too much of the Ficoll-Paque solution, and placed them in a clean 50-mL
polycarbonate tube. The tube was filled with additional SBSS, capped, and centrifuged at 350 × g for 10 min (25 °C).

Lysis of residual erythrocytes. After centrifugation, we aspirated the SBSS from the leukocyte pellet, and, without delay, lysed the erythrocytes by rapidly adding 9 mL of distilled water then briefly agitating with a vortex-type mixer to break up the pellet. Next, we immediately added 2.9 mL of NaCl (35 g/L solution), with additional vortex-mixing. Finally, we filled the tube to 50 mL with SBSS and recentrifuged, this time at 200 × g for 10 min (25 °C). The supernatant fluid was aspirated and the pellet was washed twice more with SBSS. The final pellet, about 75% lymphocytes and 25% monocytes, macrophages, etc. (as determined by Sudan Black staining and latex particle phagocytosis), was now ready for labeling, either the same day or after overnight refrigeration (4 °C).

Labeling of "B"-lymphocyte surface immunoglobulins. We mixed a pellet containing approximately 4 × 10^6 mononuclear leukocytes with 0.1 mL of a dilution of FITC-conjugated polyvalent anti-human immunoglobulin raised in a goat. The antisera dilution was prepared immediately before use, in saline (0.9 g/L NaCl), vortex-mixed, and then filtered through a 0.45 μm (av pore size) filter (Swinnex SX0001300; Millipore Corp., Bedford, MA 01730). Before use, we optimized the dilution of each lot of antisera by making a cell count on serial dilutions (usually five-, 10-, 15-, or 20-fold) and comparing the percentage of labeled cells in each. The minimum dilution yielding maximum labeling was the one we used for routine testing.

The antisera-cell mixture was incubated for 30 min in the dark at 4 °C, then centrifuged in about 5 to 10 mL of cold SBSS (200 × g, 10 min 2 to 4 °C); two additional washes followed. After aspirating the supernatant fluid, we resuspended the cells in about 0.2 mL of SBSS and placed the suspension on ice in a sealed container (to limit exposure to light). The tube with labeled cells was then placed in a covered 37 °C water bath for 30 min (the incubation period that gave maximum yield of capped cells). After incubation, a wet-mount slide was prepared and the labeled cells were observed under visible and fluorescent light (we used a "Micro-Star 1-10" fluorescent microscope with a low-energy tungsten light source, cat. no. AO 2052; American Optical Corporation, Southbridge, MA 01550).

Identification and classification of cells with surface immunoglobulin marker. Fifty cells were counted in each sample and categorized according to their membrane-protein pattern as uniform, cluster, patch, or cap. “Uniform” describes a cell surface that appears evenly shaded with lime-green fluorescence, and no distinct border. “Clusters” appear as a speckling of darkly labeled protein aggregates, outlining a distinct cell border that contains a lightly labeled (shaded) interior. “Patches” are produced as the clustering of groups of proteins migrate closer together, forming larger aggregates. In “capping,” a distinct, brightly labeled aggregate forms over one pole of a cell. Figure 1 illustrates schematically the stages through which the lymphocyte passes, culminating in capping.

Large labeled cells and weakly, partly, and passively labeled cells were disregarded, as were fluorescent debris, endocytosed cell labeling, and “clumped” groups of cells; the ability to exclude such cells is crucial to the success of the capping test for the purpose discussed in this paper. Many fields were examined alternately under fluorescent light and, to check for cell size and clumped cells, under visible light. To minimize the effect of endocytosis and to minimize fading of the fluorescence of the label after long exposure to light underground the microscope, counting should be completed within 20 min.

Only the capped cells, as a percentage of all countable cells, were recorded. All experiments were done “blind”: the microscopist was not aware of the sample source at the time of counting.

Labeling with concanavalin A. Another aliquot of the original leukocyte preparation was used for labeling with concanavalin A (Con-A; Miles Labs., Elkhart, IN 46514) in some of our patients. After we centrifuged and aspirated the supernatant fluid, we mixed the pellet with 0.5 mL of a 100 mg/L solution of fluorescein-labeled concanavalin A (diluted with SBSS and Millipore-filtered before use). The mixture was then placed on a rotator in the dark for 15 min at 4 °C, then washed twice with cold SBSS and centrifuged (700 × g) twice, 3 min each. The final pellet was resuspended in about 0.5 mL of SBSS and incubated (in the dark) at 37 °C for 30 min; from this we prepared a wet-mount slide.

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Fig. 1. Pattern of labeled cells to be counted (left) and those that should be disregarded (right)

The distribution of the fluorescent marker is not necessarily as symmetrical in the "patch" configuration as shown here. Cells as seen under fluorescent light are shown at left of each pair, and the same ones observed with a tungsten light source are shown at right.
Identification and classification of cells with concanavalin A marker. We counted 100 cells on the slide and categorized them as either uniform or capped. The fluorescent labeling looks somewhat different from that observed in the surface immunoglobulin procedure, and the microscopist should be familiar with the differences. Concanavalin A causes much clumping of cells, as well as scattered fluorescent debris—both of which can be misleading and should not be included. Again, confirmation under visible light, as described above, is necessary for accurate selection of suitable cells. In addition, fluorescence intensity deteriorates on prolonged exposure (more than 15 min).

With every batch, we included at least one control, and the counting under the fluorescence microscope was done without the counter's knowing the sample identity.

Creatine kinase determination. Creatine kinase (EC 2.7.3.2) was determined by continuous-flow analysis (SMAC; Technicon Instrument Corp., Tarrytown, NY 10591) in a modification of the method of Siegel and Cohen (13). The procedure was calibrated by the method of Rosalski (14) with a KA-150 spectrophotometer (Perkin-Elmer Corp., Norwalk, CT 06856).

Case Histories

The afflicted members of the P.E. family demonstrate a lifetime muscle weakness, with slow progression. The men in the second generation, two of them approaching the age of 60 years, can still walk, but with great difficulty (Figure 2). The 29-year-old son (III-7) of the index case, for instance, has had trouble with athletics for as long as he can remember and was on restricted duties in the army because of physical limitations. At age 27 he experienced noticeable difficulties as a small-press operator, which requires standing for many hours. On physical examination the neurologist noticed wasting of the thigh muscles, with enlarged calves; there was also some weakness of the quadriceps and hip flexors, but the Gower sign was negative. A muscle biopsy was consistent with dystrophic changes.

The proband of the D.B. family (Figure 3) had the onset of weakness at age eight or nine years, with pseudohypertrophy of the calves. He was diagnosed at the National Institutes of Health, where his serum creatine kinase activity was 180 U/L (normal, <20 U/L). He could walk until age 29, when he was confined to a wheelchair after an accident. His brother had died at the age of 34 after a similar course, after a complicating head injury. Both were free of heart disease and contracture.

The proband of the M.C. family (Figure 4) was diagnosed at age seven on the basis of supranormal creatine kinase activity and a myopathic muscle biopsy. He became wheelchair-bound at age 15, but was still active in a wheelchair at age 31, without suffering from heart disease or contracture. This patient and his two brothers were red–green color blind by the method of Gunkel and Cogan (15). Also by this method, no trace of the carrier state was found in the patient's mother or sister, but the proband's maternal aunt had color-blind male offspring. These findings suggest that the two gene loci are not closely linked, because at least one crossover must have occurred in the proband sibship. Serum glucose-6-phosphate dehydrogenase activity was measured in the family, but showed no abnormality. The proband's sister (III-2) demonstrated decreased capping on two separate occasions (26 and 34%), but serum creatine kinase and lactate dehydrogenase (EC 1.1.1.27) activities were within the normal reference interval.

Results

The clinical findings in these three unrelated kindreds were
Table 1. Capping Values, in Percent of Total Cells Labeled, for Clinically Healthy Males, Hemizygotes, and Carriers of the Becker Trait

<table>
<thead>
<tr>
<th>Age, yr</th>
<th>% Capping</th>
<th>Sig^a</th>
<th>Con-A^b</th>
<th>CK Activity, U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Healthy males in three Becker-trait families and one control</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>P.E.</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>III-8</td>
<td>28</td>
<td>52</td>
<td></td>
<td>41</td>
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<tr>
<td>II-8</td>
<td>50</td>
<td>62</td>
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<td>III-12</td>
<td>20</td>
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<td>III-10</td>
<td>27</td>
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<td>III-15</td>
<td>27</td>
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<td>4</td>
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<td>IV-1</td>
<td>7</td>
<td>54</td>
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<td>110</td>
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<tr>
<td>IV-4</td>
<td>2</td>
<td>48</td>
<td></td>
<td>53</td>
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<tr>
<td>Control</td>
<td>28</td>
<td>56</td>
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<td>73</td>
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<td>II-6</td>
<td>50</td>
<td>60</td>
<td></td>
<td>25</td>
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<tr>
<td>D.B.</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>II-2</td>
<td>70</td>
<td>54</td>
<td>20</td>
<td>53</td>
</tr>
<tr>
<td>IV-2</td>
<td>7</td>
<td>58</td>
<td>18</td>
<td>104</td>
</tr>
<tr>
<td>M.C.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II-1</td>
<td>55</td>
<td>56</td>
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<td>85</td>
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<td>16</td>
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<td>III-2</td>
<td>28</td>
<td>56</td>
<td>22</td>
<td>80</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td>56</td>
<td></td>
<td>68</td>
</tr>
<tr>
<td>Range (n = 15)</td>
<td>48-82</td>
<td></td>
<td></td>
<td>25-145</td>
</tr>
</tbody>
</table>

B. Hemizygotes

| P.E.   |           |       |         |                 |
| III-7  | 29        | 36    |         | 1730 (41)       |
| III-11 | 23        | 24    |         | 879 (1893)      |
| III-5  | 8         | 20    |         | 10 890         |
| II-2   | 38        | 28    |         | 779             |
| II-1   | 40        | 40    |         | 491             |
| II-3   | 35        | 28    |         | 404             |
| II-4   | 59        | 40    |         | 249             |
| D.B.   |           |       |         |                 |
| III-2  | 38        | 20    | 12      | 853             |
| M.C.   |           |       |         |                 |
| III-2  | 31        | 46    | 12      | 290             |
| Median |           | 28    |         | 779             |
| Range (n = 9) | 20-46 |       |         | 249-10 890     |

C. Obligate carriers

| P.E.   |           |       |         |                 |
| II-7   | 48        | 44    |         | 70              |
| II-5   | 48        | 38    |         | 76              |
| IV-2   | 4         | 54    |         | 167             |
| (6)    | (56)      |       | (318)  |                 |
| D.B.   |           |       |         |                 |
| IV-1   | 15        | 28    | 10      | 57              |
| IV-3   | 5         | 24    | 8       | 70              |
| IV-4   | 2         | 36    | 14      | 54              |
| II-1   | 69        | 26    | 10      | 153             |
| M.C.   |           |       |         |                 |
| II-2   | 53        | 42    | 10      | 31              |
| Median |           | 37    | 10      | 70              |
| Range (n = 8) | 24-56 |       |         |                 |

* Cell-surface immunoglobulins.
^a Concanavalin A.
^b The terms "hemizygote" and "affected males" are used interchangeably.
^c As defined here obligate carriers are either mothers or daughters of afflicted individuals.

The findings in 15 clinically healthy men and boys are listed in Table 1A. Fourteen of them were relatives of individuals with Becker-type muscular dystrophy; all were free of signs for muscular dystrophy, had normal serum creatine kinase activities as expected, had either a daughter with normal leukocyte capping or a healthy father with normal capping, were sons of hemizygotes, or were unrelated other than by marriage to members of afflicted kindreds. The range of 48 to 62% obtained with the surface-immunoglobulin technique agreed with the previously reported normal limits (11).

All nine afflicted individuals listed in Table 1B had both diminished leukocyte capping and above-normal serum creatine kinase activity. The low number of capped cells obtained with the surface-immunoglobulin technique was significantly below the normal range by the run test (2x = ≤ 0.01) (17, 18). The same level of significance was observed for the considerable increases in serum creatine kinase activities in affected patients over those in normal individuals.

Creatine kinase activity was normal in all but two of the obligate carriers for Becker dystrophy (Table 1C). Capping, on the other hand, was significantly below normal limits in all the obligate carriers by the run test (2x = ≤ 0.01) and closely approximated the results in hemizygotes. The exception among the obligate carriers was the four-year-old girl, P.E. IV-2, who had normal capping but borderline high serum creatine kinase activities, possibly revealing her true genotype as a heterozygote for Becker-type dystrophy. The other exception was an afflicted individual's 69-year-old mother (D.B. II-1), who had both low capping and slightly above-normal values for creatine kinase activity for her age and gender. Three more females at risk of being carriers were identified by the capping test but not by serum enzyme analysis (P.E. III-2, III-3, and M.C. III-3), increasing the total number of obligate and presumed heterozygotes to 11.

A decrease in the number of capped cells was also observed in hemizygotes and heterozygotes if concanavalin A was used as the ligand instead of polyvalent immunoglobulin. Concanavalin A capping is very useful as a confirmatory test, but does not adequately substitute for the somewhat more reliable surface-immunoglobulin technique. Values of 14% capping or more are seen with concanavalin A in normal individuals, but heterozygotes and hemizygotes of Becker-type dystrophy are likely to overlap considerably with the normal range, as in our experience with limb-girdle muscular dystrophy (18).

Discussion

Our results imply that, because of their simplicity, assays of serum creatine kinase activity remain the method of choice for confirming hemizygosity in X-linked muscular dystrophy. Patients with the severe form of X-linked dystrophy (Duchenne's) have capping values typically between 4 and 22%, whereas our subjects with the mild Becker form had intermediate capping results, between 20 and 46% in the three

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families in this study. If the occurrence of intermediate
capping values can be confirmed in more families, this test
may be of value in the differential diagnosis between Du-
chenne-type dystrophy and the early onset of Becker-type
dystrophy. Such a differential diagnostic capability would
have a decisive impact on the planning for an afflicted child's
future, because a normal or near-normal life expectancy,
at least in some cases, may be predicted in individuals with
Becker-type dystrophy.

Unfortunately, measurements of creatine kinase activity
are not sensitive enough to permit reliable detection of the
carrier state in Becker-type dystrophy. Figure 5 shows the
distribution of creatine kinase activities by sex and age in a
normal population. The "normal" (expected) range obviously
is difficult to define, even after categorizing serum creatine
kinase activities by age and sex. Creatine kinase values for all
but one of the eight obligate carriers were within the distribu-
tion of the normal population. The only distinct exception
was the 69-year-old carrier, D.B. II-1, who had moderately
above-normal creatine kinase activities for her age and sex,
in conformity with her low capping values.

In contrast to creatine kinase measurements, the success
rate in detecting obligate carriers by the capping test was
about 90%. Our results here are comparable with our find-
ings in 14 obligate carriers of the Duchenne-type dystrophy, where
all were identified as heterozygotes by the capping technique,
but only eight by serum creatine kinase activity assays.

One of the major problems encountered in preventive ge-
netic counseling remains the reliable identification of the
carrier state for X-linked muscular dystrophy. The inherent
difficulty, as in any X-linked recessive disease, is the bio-
chemical or other variability of phenotype expression from
which one attempts to derive conclusions as to the genotype
of the woman at risk. Somatic expression itself (e.g., decreased
capping) is not necessarily under direct control of the X-
chromosome. In fact, such direct X-chromosome control over
the capping process is unlikely, because we also demonstrated
decreased capping in facioscapulohumeral muscular dystro-
phy, a disease with an autosomal dominant mode of inheri-
tance (11). On this basis, the primary defect may be several
steps removed from the observed effect. If decreased capping
is ascribable to decreased membrane-protein mobility, the
primary defect might lie in membrane-associated structures
that only secondarily affect membrane behavior. The proposal
by Hauser et al. (19), that in dystrophic individuals a shift in
leukocyte-cell population dilutes those cells capable of
capping, suggests an immunological response that is derived
from muscle necrosis. Whatever the mechanism, our data
indicate that the leukocyte capping test will correctly identify
the overwhelming majority of persons heterozygous or hem-
zygous for Becker-type muscular dystrophy.

The cases presented here demonstrate the need to evaluate
all available members of the kindred, in addition to the prob-
and, wherever possible, if a reliable interpretation of the
results is to be obtained for diagnostic and genetic counseling.
In recent observations, results of the capping test led to a
change in the diagnosis from Duchenne's muscular dystrophy
to limb-girdle dystrophy in two boys, 12 and 15 years old,
because of incompatibility of the mode of inheritance with
X-linked-type dystrophy, when the fathers and not, as ex-
pected, the mothers exhibited low capping. Re-evaluation of
one of the two family histories revealed a family history of
myopathy in the father's sibship, but the exact nature of the
myopathy could not be determined. If single patients with
Becker-type muscular dystrophy are observed without an
appropriate examination of other family members, it may be
difficult to distinguish benign X-linked dystrophy from
limb-girdle dystrophy. The above cases also demonstrate the
usefulness of the capping test for carrier detection.

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clinical information on the proband in the D.B. family. This work was
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